PLANT

GROWTH

SUBSTANCES

Edited by Folke Skoog

Plant Growth Substances

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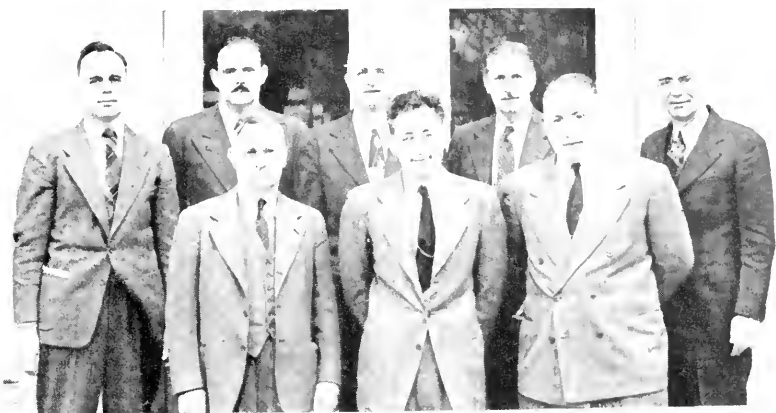
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Edited by FOLKE SKOOG

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COMMITTEE ON ARRANGEMENTS
Front row: R. H. Roberts, R. H. Burris, and J. Lederberg.
Back Row: K. P. Buchholtz, J. T. Curtis, T. C. Allen, F. Skoog, and A. J. Riker
(Missing, O. N. Allen)

Symposium meetings were held in the Wisconsin Memorial Union Theater

Publisher's Note

For bibliographical reasons, Professor Folke Skoog, chairman of the committee responsible for this book, has been designated editor. Listing volumes of essays written under separate authorship presents problems to bibliographers which do not easily lend themselves to practical solution. The Press, therefore, feels that scholars will be grateful for a simple entry under which this book may appear in files, catalogues, and bibliographies.

Preface

THE concept of hormonal regulation of growth dates back nearly a century to Julius Sachs, who deduced from his experiments on plants that special substances are responsible for the formation and growth of different organs. However, definite proof of hormonal action in plants was not obtained until 1926–28 when Went demonstrated a growth substance, auxin, in the tip of the oat seedling. This discovery marked the culmination of a long period of quantitative experimentation on the nature of plant movements and the beginning of a new approach to the study of plant growth.

Twenty years ago the work on plant growth substances was the preoccupation of a few botanists in European laboratories, who dealt with fractions of a milligram of an active material, not yet chemically defined and present in plants in concentrations too small to be detected except through its physiological activity. In this country the first small laboratory for plant growth substance research was being constructed in Pasadena by Herman Dolk, as a result of T. H. Morgan's vision and interest in the subject. From this slow beginning, the work has developed with increasing rapidity, so that it now influences all branches of botany and has had far-reaching agricultural applications.

Today several thousand persons are engaged in investigations, manufacture, and applied work on growth-regulatory compounds with physiological properties resembling those of the material originally obtained from the oat seedling. In this country synthetic plant growth regulators have become one of two main groups of organic chemicals for agricultural use. For the purpose of weed control alone nearly twenty million acres of crops were treated with these compounds during the past year.

A rapid stream of new information pertaining to the physiology, biochemistry, chemistry, and agricultural uses of growth substances is now pouring out from laboratories all over the world. To gain a perspective of the present status and progress in the field as a whole, a

committee representing different departments at the University of Wisconsin engaged in one phase or another of plant growth substance work arranged this symposium. The committee felt that, particularly since many of the persons who are responsible for the early development of the subject are still active and would be available for participation, much could be gained not only from a formal program of lectures covering principles, main lines of investigations, and recent developments, but even more from group discussions and individual contacts between persons engaged in the fundamental and applied aspects of the field.

The present volume contains the papers presented in general meetings and round table discussions, September 5–7, 1949.

The generosity of the Wisconsin Alumni Research Foundation in providing a grant through the University Research Committee of the Graduate School and of the Knapp Fund in providing a grant through the Wisconsin Centennial Committee has made this symposium possible.

On behalf of the Growth Substances Research Committee grateful acknowledgement is made to the sponsors and to all members of the University Administration and Faculty who helped to plan and conduct the meetings.

<div style="text-align: right">Folke Skoog</div>

Madison, Wisconsin, April 6, 1950.

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Plant Growth Substances

The History and Nature of Plant Growth Hormones

A. J. HAAGEN-SMIT

A LOOK at the index of the Biological Abstracts provides some indication of the general interest in the field of plant growth substances. In 1930 only fifty papers appeared, but now this number has increased to a few hundred a year.

With the growing number of papers on this subject, there has arisen a confusion in the use of the terms growth substance, growth hormone, regulator, phytohormone, formative substance, and auxin. To avoid further confusion I shall adopt Thimann's (50) recent suggestion on the nomenclature of the auxins and phytohormones, which is as follows: "An auxin is an organic substance which promotes growth (i.e. irreversible increase in volume) along the longitudinal axis, when applied in low concentrations to shoots of plants freed as far as practical from their own inherent growth promoting substances. Auxins may, and generally do have other properties, but this one is critical."

This definition excludes sugars which promote longitudinal growth when the term "low concentration" is interpreted as meaning below 0.001 molar. It also excludes nutrient salts and is intended to exclude substances such as malic and other organic acids which promote growth of the *Avena* coleoptile in a 0.001 molar concentration and less, but only in the presence of auxin. In this concept, van Overbeek's (46) criterion that an auxin should be active in the *Avena* test is replaced by a more general statement on the longitudinal growth of shoots. This change made it possible to include also substances active in other tests such as the pea test.

A phytohormone has been defined as: "An organic substance produced naturally in higher plants, controlling growth or other physiological functions at a site remote from its place of production, and active in

minute amounts" (50). This definition includes those auxins which are
of natural occurrence, certain of the vitamins, and other hormones such
as those stimulating wound growth and the postulated hormones of
flowering. It brings in certain restrictions regarding the place of forma-
tion relative to the place of action not present in the original hormone
concept of Fitting (13, 14). Since industry has recognized the sales value
of the term hormone, many synthetic preparations appear under this
name. It has, therefore, been suggested that the requirement for natural
occurrence be omitted, thereby including the synthetic substances found
to have growth effects on plants. This meeting of many plant-hormone
workers might effect some agreement as to the proper use of the terms.

The first efforts to determine the chemical nature of plant growth
stimulators were made by Fitting (13, 14) forty years ago. While visiting
the Dutch East Indies he made a study of the effects of pollination on the
orchid flower and noticed that pollen initiated swelling of the ovaries
and other phenomena of post-floration. These effects could be reproduced
by the application of dead pollen as well as by water extracts of pollen.
A test method was devised whereby pieces of cotton wool soaked in the
active extracts were brought in contact with the stigma, after which
the growth effects could be observed. Fitting made several attempts
to fractionate the pollen extract and found that the hormone was
soluble in alcohol but insoluble in petroleum ether and ether. He even
looked for sources other than orchid pollen and found that saliva caused
similar responses.

Although Fitting promised the continuation of his work, no further
data were available until Laibach (34) showed that the active pollen
substance was probably identical with one of the auxins. If Fitting had
continued his studies the history of the plant hormones might have
been quite different. After discovering the activity of saliva other excre-
tions such as urine might have been tried, and using Fitting's test
method, the auxins might have been isolated. Their parthenocarpic
effect might then have been discovered directly. Other actions such as
cell elongation in *Avena*, root formation, and the like, would have been
recognized as additional properties of the pollen hormone. There were
several reasons why history did not take this course. The microchemical
techniques were in their infancy, although Pregl at just about the same
time developed his organic microanalytical methods. The available physi-
ological tests would not have been adequate for the accuracy and the

number of routine analyses necessary for isolation of the active material. Moreover, most of these problems require research budgets, which, in the time of Fitting and long afterward, were extremely small. Fitting makes special mention of the fact that he bought some of the orchids himself. Finally, the borderline nature of the problem required the cooperation of several workers, and team work in science belongs to a much later date.

At about the time of Fitting's work, another field of plant physiology dealing with phototropic and geotropic phenomena was rapidly developing. As early as 1880 it had been shown by Darwin (9) that some influence is transmitted from the upper to the lower part in seedlings when they are exposed to one-sided illumination. Boysen-Jensen (7,8) showed in 1913 that this stimulus can be transmitted through a gelatin layer by cutting off the tips of *Avena* coleoptiles and pasting them on again with gelatin. After one-sided illumination the curvature appeared not only in the tip but also in the lower part. Boysen-Jensen was very cautious in the explanation of this phenomenon and assumed that a concentration change in the tip was responsible for this effect.

Paál (48) in 1919 demonstrated that the stimulus did not cross a layer of cocoa butter, mica, or platinum foil, and that a tip placed on one side of the coleoptile caused curvatures similar to those seen in phototropic experiments. He postulated the existence of a diffusible correlation carrier, a substance which is produced in the tip and moves downward. Phototropic effects were explained by an interruption of the normal flow of the substance through interference in its action due to some change in the protoplasm. This clear formulation of a substance as the active agent was an important step forward.

Several unsuccessful attempts were made to collect the active material from crushed coleoptiles until Went (63) showed that it diffused into gelatin from living coleoptile tips. For the measurement of the growth effect Went improved the technique introduced by Stark and Drechsel (54). This made it possible to conduct the physiological experiments on a quantitative basis, a prerequisite for the chemical isolation of the growth substance. Because of the importance for all the subsequent work let us briefly review this measuring method and its later modifications.

For this test etiolated oat seedlings are used, from which, three hours before testing, the tip is removed. After the primary leaf has been detached from its base and a second decapitation has been performed,

an agar block containing the active material is placed on one side of the cut surface of the coleoptile, the primary leaf thereby serving as a support. After 110 minutes the curvature caused by the growth difference between the two sides is measured. The curvatures are, up to a certain concentration, proportional to the amount of growth substances applied. While Went used this test primarily to measure the growth substance diffusing from tips into gelatin or agar, this method has been extended to the quantitative assay of extracts obtained by chemical procedures. This introduces complications which are often underestimated. Salt concentration, pH, and the preparation of the agar blocks have been shown to affect the curvatures. A more thorough investigation of these factors will undoubtedly increase the reliability of the results obtained. The *Avena* test has a high degree of sensitivity; 25 gammas of indoleacetic acid per liter gives a curvature of about 10°. This means that each plant receives 1/20,000,000 milligram of growth substance. A three- to fivefold increase in the sensitivity of this test was obtained by removing the seed without damaging the embryo, twelve to eighteen hours before the test (53). Straight growth measurements have also been used for the determination of the auxins and resulted in the development of the coleoptile cylinder method, whereby short pieces of the coleoptiles are immersed in the test solution. The increase in length is proportional to the logarithm of the concentration (4).

A test which has found wide application was devised by F. W. Went (66) who observed that the internodes of pea stems, when split lengthwise, curved inward when in contact with auxin solutions. The inward curvature is due to the difference in growth between the two sides of the organ, the outer side being more sensitive to auxin stimulation than the inner side. The pea test has been of great value in the testing of a number of synthetic substances, which, because of their lack of transportability, do not react in the *Avena* test. In comparing the activity of these substances it would be beneficial to apply the same rigid standardization to the pea test as Went introduced for the *Avena* test.

Many plants, among them the *Avena*, show this behavior, and it might be of advantage to use this more uniform material. Using *Avena* a greater sensitivity was obtained by splitting the coleoptile into quarters instead of halves (60). A further refinement of this method consists of removing the quarters containing the vascular bundles, which have

a somewhat lower sensitivity than the other two quarters (52). With this method indoleacetic acid can be measured in concentrations of one gamma per liter. The curvature observed is proportional to the logarithm of the concentration.

For special purposes other test methods have been developed in which the auxins are taken up in lanolin or other water-insoluble material and applied to different regions in the plant. These methods are especially useful for work outside the laboratory where the conditions cannot be as strictly controlled as is necessary for the *Avena* test.

In principle, any reaction of plants to the auxins can be used for measuring auxin activity. For example, the swelling of decapitated stems of *Vicia faba* has been utilized for an assay method (33). Hitchcock and Zimmerman (22) have made use of the fact that application of auxins changes the angle between the stem and the petiole in a number of plants.

In carrying out measurements of auxin activity all experimenters are aware of fluctuations in the sensitivity of the test object, although the temperature, humidity, and light conditions are kept constant. Measurements of the same amount of indoleacetic acid at different hours of the day gave values which may differ considerably from the average value. No explanation of this interesting phenomenon has yet been given, and attempts to prevent these variations have not been successful. In determinations where results have to be compared with those previously obtained, it is therefore general practice to test known concentrations of indoleacetic acid at the same time and express the results as indoleacetic acid equivalents. With proper technique the measurement of the over-all growth activity of active material has reached a satisfactory degree of accuracy, and the difficulties at the present time are found in the interpretation of these results.

Quantitative determination of the growth-hormone content of extracts can be seriously influenced by the presence of growth inhibitors. While the study of these inhibiting substances is of importance for understanding the mechanism of plant growth, it also has a direct bearing on the evaluation of the auxin analysis. In low concentrations of the growth substances these interfering agents may cause a positive curvature. In higher concentrations the activity curve may be considerably depressed and consequently give low values for the amounts actually present. Lipoid-soluble inhibitors have been found in extracts

of corn, tomato, radish, and other plants. In testing for the inhibiting action on *Avena* coleoptiles it is necessary to supply the plant with sufficient growth hormone so that depression of the activity can be measured. Larsen (38) measured in this way the inhibitory effects of the seed-germination inhibitors, anemonin and parasorbic acid. Effects were noticed, respectively, in concentrations of 0.1 gram per liter and of 1.0 gram per liter. The biological activity of a given amount of indole-acetic acid is neutralized in the *Avena* test by 2×10^4 to 2×10^5 times the amount of parasorbic acid. The number of known, naturally occurring, inhibiting substances is rapidly increasing, and future determination of the auxin content of plant and animal extracts must take these facts into account. It seems necessary, therefore, that in these determinations activity curves be compared with those of known growth substances. If a significant difference occurs it will then be necessary to purify the extracts before measuring their activity. Recovery experiments, whereby a certain amount of synthetic growth hormone is added to the extract to be analyzed, will also be of value. Such a procedure is to be recommended in any auxin determination where extractions have been carried out. The large amounts of solvents used to extract the exceedingly small quantities of hormone may easily introduce considerable uncertainty about the result. A routine treatment of the ether with peroxide-destroying agents is not a guarantee for the reliability of the results. A good example of the effect of inhibitors on the curvature in the *Avena* test is found in a study made by Larsen (38). The addition of a neutral extract of tomatoes depresses the curve of both auxins considerably. The acid growth substance can be easily separated from such a neutral inhibitor. However, this is not possible with the neutral auxins. To complicate matters further, inhibitors have been found which are acidic in nature. In such cases a comparison of growth curve of the pure auxin and that of the crude extract must be made in order to interpret the results correctly.

The *Avena* test made possible important discoveries in the field of geotropism and phototropism. It was shown that these phenomena depended on the presence of the growth hormone and its lateral displacement. As a direct result of the availability of a testing procedure we may also list attempts to isolate the growth substances. The first was made by Nielsen (40,41) who found that two pathogenic fungi produced in the nutrient medium substances which were strongly active

in the *Avena* test. This material proved to be thermostable, and soluble in water, ether, and alcohol. It was soon evident that auxin production was shown not only by the two fungi used by Nielsen, but also by other fungi, bacteria, and yeasts. In the isolation in Kögl's (25) laboratory in Holland several of these organisms were used as producers of starting materials, but attempts to isolate the growth substance from their media were abandoned when it was found that urine causes strong bending of the *Avena* coleoptile. After several purification steps the active agent was obtained in chemically pure form and proved to be a nitrogen-free substance ($C_{18}H_{32}O_5$).

Diet experiments showed that auxin secretion was directly connected with food intake, and when the secretion was found to be increased considerably by corn oil, this was used for isolation purposes (24). The auxin found was identical with the one from urine, but in addition a second nitrogen-free substance with auxin activity was obtained. These were distinguished by the names auxin-a and auxin-b. Using a different extraction procedure with urine, a third substance active in the *Avena* test was isolated (28). This substance proved to be 3-indoleacetic acid, a compound discovered by Salkowski (51) about 75 years before, during his investigations of protein decomposition. Indoleacetic acid was then isolated from yeast (29), and in this country Thimann (56) proved by isolation that the organism with which Nielsen had begun his studies, *Rhizopus suinus*, also produced indoleacetic acid.

During the years immediately following the discovery of the action of indoleacetic acid as a growth hormone, it was generally assumed that this substance was formed only in lower organisms. This belief was supported by its isolation from *Rhizopus* and yeast and from the evidence obtained using indirect means of identification. However, evidence gradually accumulated that indoleacetic acid could be present in higher plants too. The final proof was supplied by its isolation from wheat and corn by extraction under mild alkaline conditions (2,19), and from immature corn (18).

As a result of Larsen's (37) investigations, indoleacetaldehyde must be added to our list of naturally occurring auxins, although it is recognized that its activity is due to its conversion into indoleacetic acid in the plant.

The auxins-a and -b were shown to have the following structures (23,24). (See Fig. 1). Auxin-a is a trihydroxy acid, whereas auxin-b

$$CH_3-CH_2-CH-CH \overset{CH_3}{\underset{CH_2}{\diagup}} CH-CH-CH_2-CH_3$$

$$HC=C-CHOH-CH_2-CHOH-CHOH-COOH$$

AUXIN-α

$$CH_3-CH_2-CH-CH \overset{CH_3}{\underset{CH_2}{\diagup}} CH-CH-CH_2-CH_3$$

$$HC=C-CHOH-CH_2-CO-CH_2-COOH$$

AUXIN-b

Figure 1. Formulae for auxins-a and -b.

contains only one hydroxyl and a ketone group. The relation between the two compounds is well established since it is possible to convert auxin-a to auxin-b by the use of dehydrating agents. It has been observed that the auxins gradually lose their activity, and this change is explained by an allyl rearrangement. (See Fig. 2). The positions of the hydroxyl and carbonyl groups are such that lactone formation takes place easily. This lactone in equilibrium with the free acid undergoes, upon ultra-violet radiation, rapid conversion to an inactive product named lumi-auxone. (See Fig. 3). This conversion also takes place by irradiation in the visible range of the spectrum when carotenoid pigments are present (30). While it was not yet possible to synthesize the entire molecule, a major part of the proposed structure was confirmed by synthesis of one of the degradation products, auxin-a glutaric acid. (See Fig. 4). The synthesis is complicated by the presence of four asymmetric carbon atoms. Auxins-a and -b, with 7 and 5 asymmetry centers respectively, will present an even greater problem.

The substances isolated fulfill all the requirements necessary for activity in the *Avena* test. They are transported from the top of the coleoptile to the base in a polar fashion. It has been shown that the

AUXIN-α

$$CH_3-CH_2-CH-CH \overset{CH_3}{\underset{CH_2}{\diagup}} CH-CH-CH_2-CH_3$$
$$HC=C-CHOH-CH_2-CHOH-CHOH-COOH$$

↓

PSEUDO AUXIN-α₁

$$CH_3-CH_2-CH-CH \overset{CH_3}{\underset{CH_2}{\diagup}} CH-CH-CH_2-CH_3$$
$$H-C-C=CH-CH_2-CHOH-CHOH-COOH$$
$$\quad\overset{|}{OH}$$

Figure 2. Comparison of auxin-a with pseudo auxin-a.

Figure 3. Conversion of auxin-a to lumiauxone.

coleoptile cylinder test, and especially the pea test, does not require the transportability characteristic of substances which are active in the *Avena* test (57). When these tests are used in the isolation, substances will be found which do not possess all the qualities necessary for a true auxin, but which show the primary growth activity. Using the pea test as a guide, indican could have been isolated from urine. In plants substances like phenylacetic acid, occurring free and esterified in peppermint oil and in oil of neroli, would have been recognized as growth substances. The same is true for *cis*-cinnamic acid, which occurs free and esterified in numerous plants, the most abundant sources being Peru and Tolu balsam. These substances could well be included in the list of naturally occurring growth substances since they show the primary growth activity. Their presence in the plant could hardly fail to cause some response from the neighboring cells. In some cases where they occur in oil glands and resin ducts, we could speculate that they played a role in the cell proliferation during the formation of the glands.

As a result of the chemical investigations several substances occurring in plants and fulfilling the requirements for activity in the *Avena* test have been identified. The next question, therefore, is: which auxin
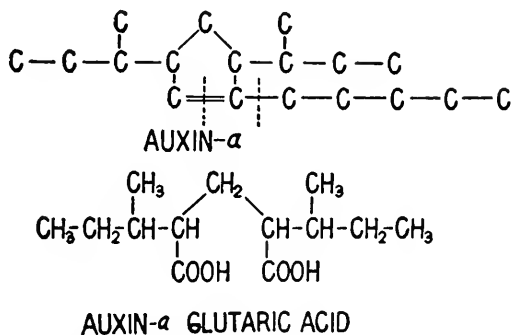
Figure 4. Formulae for auxin-a and auxin-a glutaric acid.

occurs in the *Avena* coleoptile tip and in other plants? For the identification of the auxins in plant tissues it would be preferable to isolate the active components in a pure condition. This is, of course, generally not feasible. In the case of the *Avena* tip which contains 1/50,000,000 of a milligram, it would take years to collect enough material. An additional difficulty in the case of auxins-a and -b is that both substances are converted into inactive products within a few months.

Indirect methods of determination are therefore usually the only means of answering the question of which growth substances are present in plant tissues. Went used the molecular weight determination by diffusion as a means of determining the size of the active material diffusing from the *Avena* tip. Since that time this method has served in a number of cases to decide between the occurrence of auxins-a and -b or indoleacetic acid. Went found for the tip auxin a molecular weight of 378, which was close to the molecular weight of auxin-a; but recent work has shown that when a diffusate purified by ether extraction is used, a much lower figure is found (32,71). We must accept this revision of the molecular weight with caution, since acid and alkali destruction tests were in agreement with the presence of auxin-a. It has been found in the case of the pure auxins that heating with hydrochloric acid destroyed indoleacetic acid, whereas auxin-a was resistant. On the other hand, treatment with sodium hydroxide showed the opposite effect. Auxin-b and indoleacetaldehyde were destroyed by both treatments. In the inactivation of indoleacetic acid by acid, oxygen plays an important role, and it has been shown that in a nitrogen atmosphere no destruction takes place. Since the destruction method is used extensively it should be more carefully standardized.

A third possibility of detecting the difference between the action of indoleacetic acid and the tip growth hormone is based on the phototropic behavior of plants. In the *Avena* coleoptile Went showed that with low light intensity of 20 to 100 meter candle seconds a lateral movement of the auxins takes place, causing an increased concentration at the dark side. At the same time there is a lowering of the total auxin content, this destruction being of the order of 25 per cent. If one or the other auxin is responsible for the phototropic curvature, artificial application of one of these substances to growth-hormone-free coleoptiles should give similar response to that of the intact coleoptile. This experiment has been carried out by Koningsberger and Verkaaik (31) and by Op-

penoorth (42), who found that a coleoptile cylinder supplied with auxin-a showed phototropic behavior, whereas those with indoleacetic acid did not. The destruction under the influence of light has been explained by the conversion of auxin-a-lactone to inactive lumiauxone. These changes take place rapidly by irradiation with ultraviolet light, and in the presence of both α- and β-carotene the inactivation occurs in the visible spectrum. The proposed mechanism of phototropic action finds support in the presence of carotenes in the coleoptile and in the agreement between the spectral sensitivity of the coleoptile to light and the absorption spectra of the carotenes. Recently, however, Galston (15) has shown that indoleacetic acid is easily destroyed by light when a suitable activator is present. Lactoflavin was found to have such an action, and rapid decarboxylation and oxidation is observed in vitro in sunlight, and the action spectra also agree with that of the coleoptile. Van Overbeek (44) found that the growth inhibition of *Avena* coleoptiles after exposure to light does not seem to occur when the growth hormone applied to the top of the coleoptile is indoleacetic acid instead of auxin-a.

There is a possibility that an additional method of distinguishing indoleacetic acid and auxin-a may be found in the experiments of Guttenberg (17) on deseeded, derooted coleoptiles of *Avena* and hypocotyls of *Helianthus annuus*. After one-sided application of tip growth substances, the curvatures appear during the first two hours, whereas those from application of indoleacetic acid take from 3 to 10 hours to develop. This slow reaction of indoleacetic acid is explained as being due to the production or release of auxin-a under the influence of indoleacetic acid. Further support for this theory is found in experiments whereby auxin-free coleoptiles are treated on the outside with indoleacetic acid. After extraction, the extracted growth substance behaves like auxin-a in acid and alkali stability tests.

Another method which holds promise in distinguishing between the auxins could be based on enzymatic destruction. Thimann (58) observed a considerable loss in activity when auxin was incubated with leaf extracts of *Vicia faba* and *Helianthus* and attributed this effect to the enzymatic destruction of the auxin. The importance of this phenomenon for the regulation of plant growth was shown by van Overbeek (43), who found that dwarf corn contained greater than normal amounts of this destructive agent. Larsen (36) made some steps toward purification of the auxin inactivating substance and found it to be of enzyme nature.

Tang and Bonner (55) showed that the optimum range of activity fell between *p*H 6.2 and 6.7. It is reported that the enzyme does not attack indoleacetamide, indolebutyric acid, indolepropionic acid, indolecarboxylic acid, or tryptophan, and therefore seems to possess a considerable degree of substrate specificity.

It would be desirable to have chemical methods to determine quantitatively the different auxins. Up till now such methods have been developed only for indoleacetic acid. Salkowski (51), analyzing the products of putrefaction of proteins, found that one of the products formed gave a red color with nitrite or ferric chloride and a mineral acid. Both reactions have been carefully studied (39) and the optimum conditions for the reactions determined. Methods were developed whereby indoleacetic acid can be determined over a range of 5 to 200 gammas per milliliter. Especially recommended is the nitrite method, where the coefficient of variability is less than three per cent. The ferric chloride reaction has often been used for the identification of indoleacetic acid in plant material, as, for example, in the media of fungi. Wildman and Bonner (70) found that a considerable portion of the *Avena* tip growth activity could be explained by the presence of indoleacetic acid.

After discussing the question as to which auxins occur in plant tissues, another problem connected with their production, storage, transport, and action presents itself. It had been observed that a part of the auxins is readily extracted from the tissue, but that considerable additional amounts of auxins could be obtained by continued ether extraction or by treatment with hydrolytic agents. Thimann and Skoog (61) have shown that many ether extractions spaced over several months are required to extract all the ether-soluble auxins present in *Lemna*. They concluded from their experiments that the continued production of auxin in ether was due to an enzymatic liberation from an inactive form, probably a protein. These observations have since been confirmed by a number of investigators. Determination of free auxins prevents this enzymatic production by boiling previously frozen and ground material, or by carrying out the ether extraction at zero degrees.

Wildman and Bonner (69) obtained from spinach leaves a fraction which is homogeneous in ultracentrifugation and electrophoresis experiments and has phosphatase action. This fraction gave, on treatment with alkali or proteolytic enzyme, a growth hormone which is probably identical with indoleacetic acid. The bound auxin seems a part of the

broken molecule since repeated fractional precipitation of the protein with ammonium sulfate did not remove the auxins.

In seeds free and bound auxins have also been shown to occur. In corn oil some of the auxin can be readily extracted with water, and consists mainly of auxins-a and -b. Hydrolysis of the oil gives additional quantities of auxins which are probably present in the ester form. When the entire seed of cereal grains is treated with dilute alkali, amounts of auxins are found which are several times greater than those available from seeds after extraction with water. This liberated auxin was isolated and shown to consist mainly of indoleacetic acid (2,19).

As Gordon and Wildman (16) have shown, some of this liberated auxin has its origin in tryptophan, which releases under mild treatment small quantities of indoleacetic acid measurable in the *Avena* test. It is, for example, unstable at 37°C. at pH 10.5, or in contact with cold phosphate buffer at pH 4.6. Even melting with agar releases some growth hormone. Similar degradations of tryptophan take place within the plant, and it has been shown that an enzyme is responsible for this conversion.

These findings do not invalidate all conclusions based on previous auxin extraction methods. However, they must be considered in future work. When, for example, wheat or corn is treated at a pH of 10.5 for two days, an increase of 5 milligrams per kilogram in the auxin content is noted over the readily extractable auxin. As Avery and Berger (1) have shown, it is safe to conclude that this amount represents auxin in bound form and is not due to the conversion of proteins containing tryptophan. Avery and Berger purified the auxin complex and found it to be protein-like. Skoog (53) has shown in the *Avena* that inactive precursors originating in the seed travel upwards and are able to induce growth of the coleoptile after 2 to 6 hours. It is possible that the auxin complexes mentioned serve as the auxin reserve for the plant. Since Wildman *et al.* (71) discovered an enzyme which converts tryptophan to indoleacetic acid we have to include this substance and tryptophan-containing proteins as potential sources of auxins in the plant.

The proportion of free to bound auxin varies greatly in different plants and plant parts. In *Lemna* only two per cent is free according to Thimann (59), whereas the free auxins in *Avena* coleoptile tips account for nearly all of the total auxin. The study of the relative proportions of free and bound auxin is of importance for the understanding of several growth

phenomena. For example, van Overbeek *et al.* (47) have shown that the geotropic response of horizontally placed sugar cane is due to the formation of free auxin at the expense of the bound form in the lower side of the node.

In a search for growth factors other than auxins the effect on the seedling of removal of the endosperm was studied. Even after supplying sugars and mineral salts the growth of young seedlings is then greatly retarded. In experiments on pea seedlings it was shown that when a cotyledon was placed in the nutrient medium together with the embryo considerably better growth is obtained. Since it was known that several vitamins were present in the endosperm these were tried, and it was found that biotin, vitamin B_1, ascorbic acid, and even the animal hormone, estrone, caused increased growth of the seedlings over the controls. The addition of thiamin was characterized by a greatly increased root system (26). This effect is also observed in isolated roots, and Bonner (5) has shown that vitamin B_1 is a major factor in the development of the root, and that its function is supplemented by pyridoxine and probably nicotinic and pantothenic acids (6). The material diffusing from the cotyledons is apparently rich in growth substances, a conclusion substantiated when pea diffusate was found to be active in promoting leaf growth. Using the growth increase of circular discs of tobacco and radish leaves as a test method, adenine was isolated and recognized as one of the active agents. Embryonic pea leaves also showed increased growth under the influence of adenine. For this tissue, therefore, adenine acts as a typical plant hormone (3). Some investigators have found that the auxins also affect leaf growth. Went (65) has given the name rhizocaline to the complex of chemical factors other than auxin which are involved in root formation. They are found in buds and cotyledons and are formed by leaves in the presence of light. The nature of these substances is unknown. The same is true of caulocalines, factors which influence stem growth and are produced in roots, and the postulated flower hormones.

We are better informed regarding the nature of a different type of growth activator: the wound hormones. Upon injury of a plant the intact cells surrounding the wound show a greatly increased rate of division. Wiesner (68) suggested in 1892 that special substances are probably produced by wounded cells. Haberlandt (20,21) found that the surface of potato tuber responded to the application of phloem tissue and to crushed cells or their extracts. Similar phenomena can be studied on

F. W. Went, left, and A. F. Blakeslee

C. A. Elvehjem, left, and A. J. Haagen-Smit and Kenneth V. Thimann

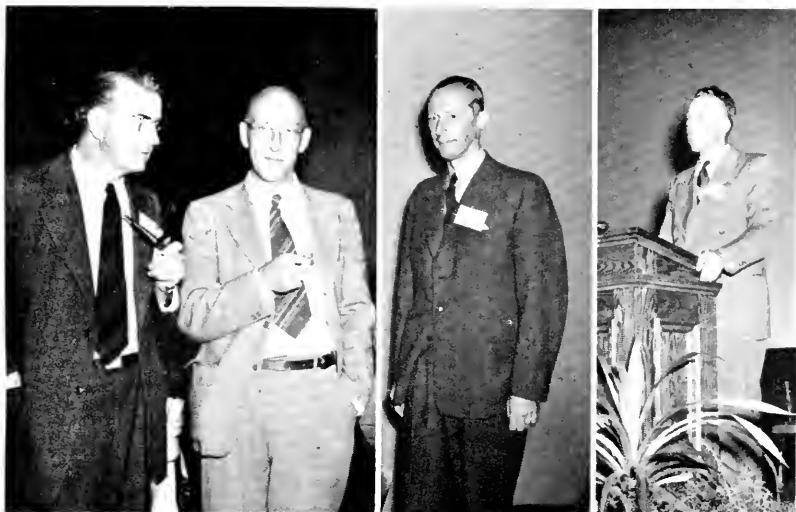R. S. Dunham, K. C. Barrons, and K. P. Buchholtz

Kenneth V. Thimann, George S. Avery, Jr., Frederick G. Smith, and
R. H. Burris

R. H. Roberts, left, and Felix G. Gustafson

J. van Overbeek, left, and P. W. Zimmerman

Left, R. Alexander Brink and E. L. Tatum; center, Hans Burström; and right, Esmond E. Snell

O. N. Allen, R. S. de Ropp, Albert C. Hildebrandt, T. C. Allen, A. J. Riker, and F. H. Newcomb

other plants. Wilhelm (72) introduced a test method using the response of the parenchyma tissue which lines the hollow stem of the Windsor bean, and Wehnelt (62) developed a test using the lining of the kidney bean pod. A drop of crushed tissue applied to this layer causes rapid cell division, and a small intumescence about one to two millimeters high appears. The height of this new tissue measured after 48 hours is to a large extent proportional to the concentration of the wound hormone. The beginning of the curve represents reactions nonspecific in nature, such as the result from application of water, strong sugar solutions, and toxic substances. After fractionation of the bean pod juice, the active material proved to be an α, β-unsaturated dicarboxylic acid of twelve carbon atoms (11). This 1-decene-1,10-dicarboxylic acid has been named traumatic acid, and is active in amounts as little as 0.1 gamma in the bean pod. Since in growth a considerable expenditure of building material as well as stimulatory substances are necessary, it is not surprising that several cofactors can increase the activity of traumatic acid. Most striking is the effect caused by glutamic acid, which enhances the activity of the wound hormone about ten times.

English (12) has prepared several analogues of traumatic acid, and shown that the activity is confined to the dicarboxylic acids. Both saturated and unsaturated acids are active, but the double bond, while not essential, increases the hormone action. For example, decane-1,10-dicarboxylic acid has only half the activity of traumatic acid. The structure of traumatic acid seems to indicate that it is a result of the breakdown of fatty acids or their derivatives such as lecithins and fats. No efforts have been made to establish its origin, and there is no information on its mode of action.

Since the time that the hormone concept was introduced in plant physiology considerable progress has been made. Some of the correlation carriers have been isolated and their actions have been studied. Still many of the hormones remain to be discovered, and in addition a great number of new problems have appeared. In striving for simplification deductions in this field are often based on the results of work on a variety of plants all through the plant kingdom. These extrapolations have had a stimulating effect on the development of the hormone field, but with the increase in our knowledge it is evident that there are a large number of individual problems which require special investigation. Consequently, the complete picture of hormone action on plant growth will un-

doubtedly be a complicated one. If we consider that the important applications in agriculture are due to the exploitation of only one of the hormones we can expect a great deal from the future.

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The Synthetic Auxins: Relation Between
Structure and Activity

KENNETH V. THIMANN

THE discovery of the auxins in the early 1930's was accompanied by a great increase in physiological experimentation. This broadened the basis of our knowledge of auxins, for it brought to light the fact that the auxins control many activities of the life of plants. In addition to promoting cell elongation of stems and coleoptiles, which was the first known function, the following processes (reviewed in 10) are all controlled or promoted by auxins: 1) the initiation of roots on stems; 2) the initiation of roots on roots (but only to a limited extent); 3) cell division both in the cambium and other stem tissues; 4) growth of the ovary into a fruit (involving both cell division and enlargement); 5) growth of parenchymatous tissues into tumors; 6) streaming of protoplasm (studied only to a limited degree). However, the development of buds and the elongation of roots are inhibited by auxin in all but the very lowest concentrations.

The first widely held view of auxin action was that it acted in some way to increase the plasticity of the cell wall, thus allowing elongation to take place under the cell's own osmotic force. However, the participation of auxin in so many processes, involving also cell division and the inhibition of enlargement, makes it clear that this concept is insufficient and that some much more profound action or group of actions must take place. This leads at once to the idea that auxin probably acts in some process of metabolism common to nearly all plant cells and capable of leading to varied results depending on the influence of additional factors on the cell. Such a process of metabolism would of course be enzymatic. Since the amount of auxin needed is far too small for it to

be a substrate it must act catalytically and thus in some way become a part of some enzyme system. This of course raises the question as to what enzyme systems are involved in growth. In addition, however, the participation of a small molecule in an enzyme system presupposes special properties in that molecule. Hence this idea focuses attention on the nature of the auxin molecule itself; what are the properties of the substance—a relatively simple molecule—which might enable it to act in an enzyme system? We are in a position to attempt an answer to this question now because of the large number of synthetic auxins that have been prepared.

In other comparable cases, like those of the coenzymes and prosthetic groups, there are strict limitations of structure, and quite small changes in the molecule such as, for example, the substitution of two ethyl for two methyl groups in pyridoxine, make the compound inactive. In other cases, such as the replacement of the thiazole ring by a pyridine ring in thiamin, a relatively small change may make the compound a competitive inhibitor. The case of the auxins is somewhat different since even the first known compounds, the naturally occurring auxins, show wide differences in structure and appear virtually unrelated. However, if we consider auxin-a (I) and indoleacetic acid (II) more carefully it will be seen that in fact they have many properties in common. In the
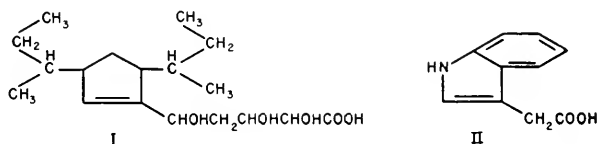
first place, both have an unsaturated 5-membered ring with an acid group situated at a distance from the ring. Second, the presence of the N atom in the ring of one compound but not in that of the other indicates that this atom is not essential for activity. A study was therefore made of the activity of compounds resembling indoleacetic acid but without the nitrogen. It was found at once (9) that indeneacetic acid, (III) is highly active; the ring N atom is therefore not important for activity. However, III shows some curious properties. In the *Avena* test it gives
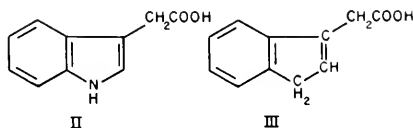
good curvatures, though with only about 1 per cent of the activity of indoleacetic acid, but the curvatures are limited to a very short apical zone. In growth tests in which the plant parts are immersed in the solution, the activity is much higher relative to that of indoleacetic acid than in the *Avena* test, reaching 20 per cent in the curvature of slit pea stems. Further, in the formation of roots on pea stems III has low activity when applied to the apex of the stem but is highly active when

IV   V
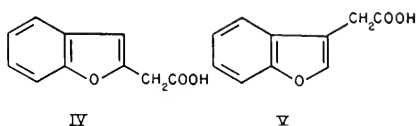
applied to the base; the difference is of the order of 100 times. (In later studies we have found this compound excellent for the rooting of woody cuttings, when applied to their bases.) These observations show that the activity of III is being limited by the ability of the plant to transport it. A similar analysis of the activity of benzofurane-2-acetic acid (IV) gave comparable though more extreme results. It is completely inactive in the *Avena* curvature test but active on immersed sections; and in root formation it is inactive when applied to the tip but active at the base. Benzofurane-3-acetic acid (V), the spatial analogue of II, is about twice as active as IV but is also limited by transport.

Activity in inducing growth thus depends not only on activity of the compound per se, but on its ability to be transported in the plant. It is obvious that there may be many other such secondary characteristics which influence the apparent activity of a compound. The following may be suggested: 1) ability to enter the cell (solubility in the plasma membrane); 2) resistance to inactivation by plant enzymes; 3) stability to light (in certain tests), or to the combination of light and photodynamic substances like eosin, riboflavin, or carotenoids; 4) dissociation constant of the acid group, since the anion is in general less active than the undissociated acid.

The influence of many such factors can be minimized by using a short-time test with a single standard tissue immersed or floating in a solution of known pH in darkness. The curvature of slit halves of etiolated pea stems has been most widely used and most of the conclusions which follow are based on this test. To avoid curvatures due to release of residual auxin in the tissues, the slit halves are placed in water for two hours before exposure to the test solution (16).

Structural Requirements for Primary Activity.—Interpreting the pea test as indicative of primary growth activity, a great many substances have been prepared and tested. In general it appears that only compounds with an unsaturated ring system are active. The saturated compounds cyclohexaneacetic and decahydronaphthaleneacetic acids are both inactive. In the *Avena* test, even saturation of the heterocyclic double bond of indoleacetic acid abolishes activity (5). Further, all the active compounds are acetic, propionic, or butyric acid derivatives. Examples are naphthalene-1-acetic acid (VI), anthraceneacetic and even phenylacetic acid, but not cyclohexaneacetic acid. The salts are in general less active than the acids, though at the pH of the cell (5.5–6.0) they are partly converted to the free acid. Acids stronger than indoleacetic acid (pK 4.75), however, are more fully dissociated, and their apparent activity should be corrected for the extent of dissociation (1). Esters of the acids are in general active, and methyl esters have about the same activity as the acid; ethyl esters, generally less. In the *Avena* test Kögl and Kostermans (5) found that activity decreased with the increasing size of the alkyl esterifying group and concluded that the esters must be hydrolyzed to the free acids to produce growth. It is probable that such hydrolysis is only necessary for transport and that, for primary activity, the ester is active per se. Amides present a similar problem. In the case of naphthaleneacetamide, although the activity is lower than that of the free acid, the curve relating activity and concentration is quite different from that of the acid (11), whereas if hydrolysis were involved one would expect the two curves to be parallel. Further, at the optimum concentration no trace of ammonia could be detected in the solution (11). It is probable, therefore, that amides too are active per se for primary activity. In one or two cases nitriles show activity, which can safely be ascribed to hydrolysis to the acid.

Spatial configuration evidently plays an important part in determining activity. In phenylacetic acid (VIII), substitution of one of the side-chain hydrogen atoms by a methyl group (IX), or of both of them by a methylene group (X), does not appreciably change the activity,* but substitution of them by two methyl groups (XI), abolishes it completely (3). This suggests steric hindrance and indicates that the carboxyl must bear a certain spatial relation to the ring. Clear evidence for this is given

*The *n*-propyl and allyl derivatives are also active, both being more active than phenylacetic acid, though the isopropyl derivative is almost inactive (14).

CH₂COOH → CH_2COOH

Structures:

VI — naphthalene with CH_2COOH side chain

VII — naphthalene with $CH=NOOH$ side chain

VIII — benzene with CH_2COOH

IX — benzene with $CH-COOH$ and CH_3

X — benzene with $C-COOH$ and CH_2

XI — benzene with C bearing CH_3, CH_3, and $COOH$

XII — benzene with $CH=CH$ and $COOH$

XIII — benzene with $CH=CH$ and $COOH$
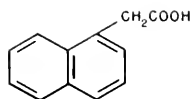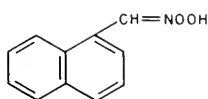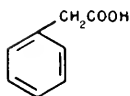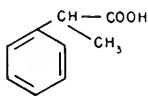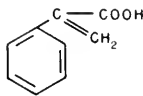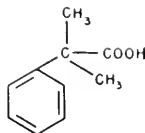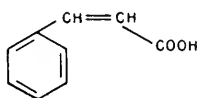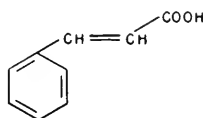
by the cinnamic acids; the *cis*-form (XII) is active and the *trans*-form (XIII), inactive. The *p*-methyl and the *o*-methoxy derivatives of these two acids behave similarly.

Putting all these facts together, Koepfli, Thimann, and Went ten years ago (3) concluded that the minimum structural requirements for primary activity are: 1) a ring system as nucleus; 2) a double bond in this ring; 3) a side chain; 4) a carboxyl group (or a structure readily convertible to a carboxyl) on this sidechain at least one carbon atom removed from the ring; and 5) a particular space relationship between the ring and the carboxyl group.

The term carboxyl should be broadened, however, because of the activity of naphthalene-1-nitromethane, which in the *aci*-form (VII) has an acid group isosteric with a carboxyl (13). Indican also has weak activity (13), though no sulfonic acid yet tested is active. It may thus be that the requirement is merely for an acid group which is not too highly dissociated.

The Ring.—The nature of the ring is important. As a rule 5-membered rings do not confer activity. Pyrroleacetic acid, in spite of its resemblance to the indole structure, is inactive, as are the acetic derivatives of imidazole, furane, and thiazole. Neither Δ¹-cyclopenteneacetic nor Δ²-cyclopenteneacetic acid is active, which is of interest in comparison with auxin-a (I), (11). The fusion of a 6-membered ring with the 5-

membered ring confers activity, however, as shown by the acetic deriva-
tives of indole, benzofurane, and thionaphthene. The activity of auxin-a
and -b seems exceptional, but if we consider formula I it might be
suggested that the secondary butyl group has almost the configuration
of a ring. This phenomenon, of course, is well known in the case of the
estrogens, where the substituted derivatives of stilbene reach a maximum
activity with the diethyl derivative (XIV), which most nearly resembles
estradiol (XV). Further studies with alkyl-substituted 5-membered rings
are desirable to clear up this point.

The need of unsaturation in the ring is characteristic and cannot be
replaced by unsaturation in the side chain. Thus Δ^1-cyclohexeneacetic

XIV   XV
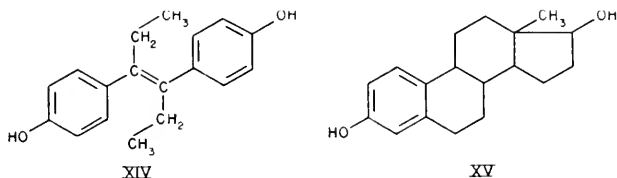
(XVI) is active, but cyclohexylideneacetic (XVII) is not (3); indene-3-
acetic (III) is highly active, but benzofulvenecarboxylic (XVIII) is very
weak (11), although, of course, it retains unsaturation in the benzene
ring. It is not that unsaturation in the side chain destroys activity, for
cis-cinnamic acid(XII) and atropic acid (X) are active. Thus, irrespective
of the degree of saturation of the side chain, there must be unsaturation
in the ring itself. Veldstra in 1944 (13) brought forward evidence that
the unsaturation of the ring is needed to confer interfacial or surface
activity upon it. This evidence depends on the behavior of a series of
substances when their current-voltage curves are determined with the
dropping mercury electrode in acid methanol solution. Under these
conditions the control curve passes through a sharp maximum, ascribed
to oxygen adsorbed on the cathode, while active auxins strongly depress
this maximum. Seven active compounds behaved in this way while
seven inactive ones had no such effect. Eight other inactive compounds
did depress the maximum, however. Veldstra concludes that the ring
must have strong surface activity, that is, it must be capable of ad-

XVI   XVII   XVIII   XIX
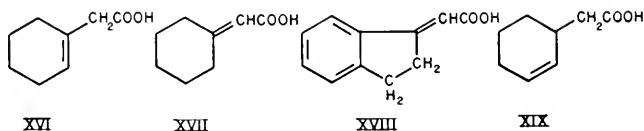
sorption to some reactive surface, a property essential for activity but of course not sufficient by itself. Such a view agrees with the concept of a coenzyme or similar function discussed above. It should, however, be pointed out that Δ^2-cyclohexeneacetic acid (XIX) is inactive, (compare the activity of XVI) from which it seems that the double bond must be adjacent to the side chain (3,17), as indeed it is in all the other active substances. This would suggest that the adsorption to a surface may not be as unspecific as it sounds and may require very definite configuration.

The Side chain.—The acid group must be separated from the ring. In the *Avena* test (with the indole ring) the one methylene group is sharply optimal, but for primary activity the butyric acid with 3 methylene groups has about the same activity. The propionic and valeric derivatives have definitely less activity in all tests, and this holds both for indole and for naphthalene compounds.

There are one or two cases of carboxyl groups attached directly to the ring which must be considered. It was claimed that 2-bromo-3-nitro-benzoic acid was active, but its action appears to rest on synergistic effects rather than true primary activity. Veldstra and van de Westering (14) have kindly made available their studies on the naphthoic acids;
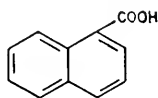
XX   XXI   XXII
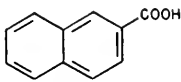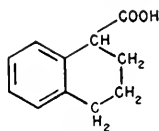
the α-acid (XX) apparently has real but slight activity, while the β-acid (XXI) is inactive. However, hydrogenation of the substituted ring (XXII) increases the activity 5–10 times. It may be pointed out that in these compounds the COOH is attached to one ring but may be considered separated from the other, that is, the compounds may be thought of as derivatives of phenylacetic acid. This is particularly true when the ring is hydrogenated. It is significant that the 5,6,7,8-tetrahydro-derivative has very little if any activity, and this compound is of course analogous to cyclohexane-1-acetic acid. The activity of naphthoic acid will be referred to again below.
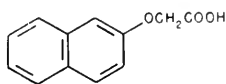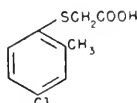
XXIII   XXIV

A heteroatom may take the place of carbon in the side chain, as in naphthoxy-2-acetic acid (XXIII), with an oxygen atom, and 2-methyl-4-chlorophenylthioacetic acid (XXIV), (11) with a sulfur atom, which is highly active. (See Table 1.) Phenoxyacetic acid and phenylthioacetic acid, however, are inactive (10), while phenylpropionic acid has a little activity, so that the oxygen and sulfur atoms are less effective than the carbon. Phenylglycine with a nitrogen atom is also inactive.

A striking effect is caused by introduction of a hydroxyl into the side chain. The activities are as follows (compared to indoleacetic acid as 100):

Parent Compound		Hydroxy Derivative	
Phenylacetic	10	Mandelic	0
α-phenylpropionic (XXV)	6	Atrolactic (XXVI)	0.5
		Tropic (XXVII)	0
Indeneacetic (III)	20	Indeneglycolic ethyl ester (XXVIII)	0
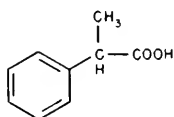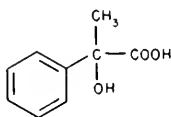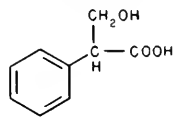
XXV   XXVI   XXVII
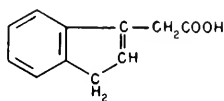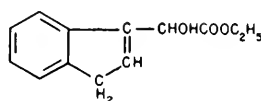
III   XXVIII

Recently Went has reported (17) that mandelic and tropic acids are also virtually devoid of preparatory activity (see below). Thus the hydroxyl in the side chain essentially destroys all activity. An original explanation will be offered below for this clear-cut effect. It must be noted, however, that auxin-a with its 3 hydroxyls is again an exception.

The Problem of Spatial Configuration.—To the cases of the cinnamic acids and the chain-substituted phenylacetic acids many interesting observations have been added. Unfortunately their meaning is not yet clear.

There are now three cases in which optical isomerism influences activity. The optical isomers of indole-α-propionic acid (XXIX) were tested by Kögl and Verkaaik (6) in the standard *Avena* test. The (+)

acid was found to be 30 times as active as the (−). On straight growth of immersed sections, however, the two isomers have the same activity.

XXIX

XXX
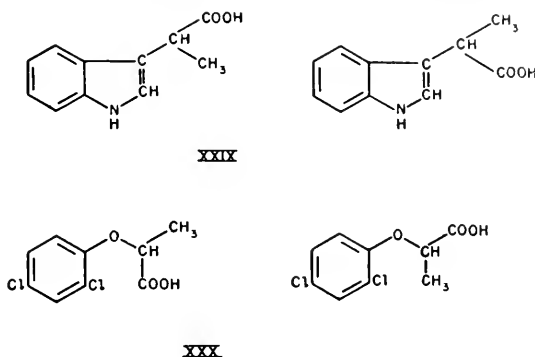
The difference is not one of primary activity therefore, a fact which was confirmed by direct demonstration of a difference in transport rate and in rate of inactivation by tissue brei. The isomers of 2,4-dichlorophenoxy-α-propionic acid (XXX) present a different picture. In this case the d- and racemic acids* show differences in the pea test, the d-form being almost exactly twice as active as the racemic, which means that the activity of the l-form is very low (11). This, then, is a difference in primary activity. The third case is that of the 1,2,3,4-tetrahydro-naphthoic acids (XXII) of which the (−) form has nearly the activity of indoleacetic acid, while the activity of the (+) form is very low; this again refers to activity in the pea test (14). Optical isomerism, therefore, does influence primary activity.

The geometrical isomerism is perhaps more interesting because it has given rise to a consistent theory. Veldstra (13) has pointed out that in trans-cinnamic acid the dipole of the carboxyl is held in the plane of the ring, while in the cis-isomer it is held at an angle to it. He has thus generalized the five requirements of Koepfli, Thimann, and Went to two, namely, "a non-polar part (ring-system) active interfacially, carrying an acidic polar group (preferably a carboxyl group) in such a spatial position with respect to the nucleus that this group is situated out of the ring system as far as (or as frequently as) possible" (14). To the cinnamic acids, tetrahydronaphthylideneacetic (XXXI) and α-naphthaleneacrylic (XXXII) have been added. In each case one form is active and the other

*Kindly supplied to me by Dr. Franklin Jones.

CHCOOH   HOOCCH

CH₂   CH₂
CH₂   CH₂
H₂   H₂

XXXI

H   COOH

H—C=C—COOH   H—C=C—H

XXXII
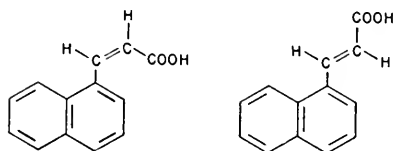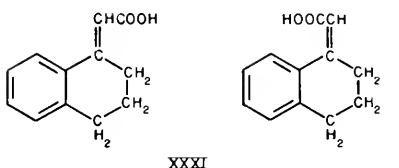
not, and there is evidence from the ultraviolet absorption spectra that the active form of both has the *cis*-configuration (2). The surface activity, determined by the polarographic procedure, is the same for the two isomers of XXXI. A detailed criticism of Veldstra's theory would be out of place here, but at least in the case of XXXI the argument is open to doubt. For, according to principles of organic chemistry, derived from study of tetramethylethylene and other simple cases, when a carbon atom forms a double bond the other two bonds lie in the same plane. Hence in both forms of XXXI the carboxyl would be expected to lie in the plane of the ring. Because the right-hand ring is saturated, however, it can be somewhat buckled, raising carbon atom number 2 above the plane of the ring and hence making it possible for the COOH to lie slightly below the plane, and thus to avoid interference with the hydrogen atom on the aromatic ring. Judging from models, the effect is very small.

The same theory has been applied to the nitrophenoxyacetic acids. Of these only the *m*-isomer shows appreciable activity. It is pointed out by Veldstra that the *o*- and *p*-isomers are capable of resonance with a quinoid structure, in which the oxygen atom would be held in the plane of the ring. This restricts freedom of rotation and favors positions in which the acid dipole is at an angle to the plane of the ring. But careful examination of scale models shows that this restriction would in fact hold the carboxyl at least as much out of the plane of the ring as in it, that is, the *o*- and *p*-compounds should have at least as high activity.

Another more general consideration opposes this view also. If the maintenance of the carboxyl out of the plane of the ring were the princi-

pal requirement for activity then a compound like *cis*-cinnamic acid, in which this position is fixed, should be much more active than, say, indole-acetic acid, in which this is only one of many positions attainable by free rotation.

The effect of ring substitution can be considered in this connection. Introduction of a methoxy group into the benzene ring of indolepropionic acid, in any one of 3 positions, 4, 5, or 6,* inactivates it completely (3). On the other hand introduction of halogens into the ring of phenyl derivatives greatly increases activity. The phenoxyacetic acids show the following primary activities (10) (relative to that of indole-acetic as 100):

Phenoxyacetic	ca.	0
o-chlorophenoxyacetic		4
p-chlorophenoxyacetic		200
2,4-dichlorophenoxyacetic		1000
2,4,5-trichlorophenoxyacetic		500

Similarly, 2,4-dichlorophenylacetic acid has very high activity. A striking case is that of phenylbutyric acid whose activity is virtually zero; *p*-bromination raises it to about 15 per cent of that of indoleacetic acid (see Table 1). Methyl groups in the *o*- and *p*- position apparently increase the activity in the phenoxyacetic series about as much as chlorine atoms (7).† Many similar examples can be drawn from the work of Norman and his colleagues at Camp Detrick, though they used different tests.

It is difficult to see how substitution in the remote para- position could twist the carboxyl out of the plane of the ring, or how in indoleacetic acid substitution of a methoxyl in the remote 5-position could have the opposite effect. According to Veldstra the influence of the halogens may be exerted on the lipophilic properties of the molecule, which are increased thereby; however, if the molecule becomes too lipophilic the balance between this and its hydrophilic properties is disturbed

*On the other hand, S. P. Findlay and G. Dougherty [*J. Biol. Chem.*, 183:361 (1950)] have recently reported, but without quantitative data, that 5-, 6-, and 7-methoxyindoleacetic acids are active. The activity is probably rather low. Their data also confirm the inactivity of $\triangle^2$-cyclopenteneacetic acid. (See page 25).

†R. M. Muir, C. H. Hansch, and A. H. Gallup [*Plant Physiol.*, 24:359–366 (1949)] have recently reported in some detail on the halogenated phenoxy compounds.

TABLE 1

List of compounds whose activity is here quantitatively compared for the first time

Name	Activity* (Indoleacetic acid = 100)
Benzofulvenecarboxylic acid	0.5
Benzofulvenehydroxycarboxylic acid	0
Indene-3-glycolic acid ethyl ester	0
Phenylthioacetic acid	0
2-methyl-4-chlorophenylthioacetic acid	200
Methylbenzoquinonethioacetic acid	0.2
Trimethylbenzoquinonethioacetic acid	0
Phenoxyacetic acid	*ca.* 0
2-chlorophenoxyacetic acid	4
4-chlorophenoxyacetic acid	200
2,4-dichlorophenoxyacetic acid	800–1,200
2,4,5-trichlorophenoxyacetic acid	500
2,3,4,6-tetrachlorophenoxyacetic acid	1
Pentachlorophenoxyacetic acid	*ca.* 5
Bis-(2,4-dichlorophenoxy)-acetic acid	1.5
2,4-difluorophenoxyacetic acid	12
2,4-dichlorophenylacetic acid	15
Racemic 2,4-dichlorophenoxy-α-propionic acid . .	600
D-2,4-dichlorophenoxy-α-propionic acid	1,200
1,4-naphthoquinone-2-butyric acid	0
4-bromophenylbutyric acid	15
4-hydroxyphenyl-γ-ketobutyric acid	0
Naphthalene-1-acetamide (approx.)	10

*Based on slit pea stem curvatures.

and activity is reduced (15). It seems to the present author that the evidence for this conception is far from complete though it is in principle reasonable.

Enhancement of Auxin Action.—To complete the factual picture one other phenomenon should be mentioned. Many compounds, inactive by themselves, increase the apparent activity of the auxins. Went, who discovered this phenomenon, studied it by pretreating the pea sections with the inactive substance and subsequently placing them in a low concentration of the auxins (16). He views the growth process as taking

place in two steps, the first or preparatory reaction being pH insensitive and being capable of accomplishment by a wider variety of structures than the growth reaction can be. In general, substances which have all but one of the structural requirements of an auxin have preparatory activity (17), and these are called hemi-auxins. They include 2-bromo-3, 5-dichlorobenzoic acid (carboxyl adjacent to ring), cyclohexaneacetic acid (saturated ring), thiazoleacetic acid (5-membered ring) and vinyl-acetic acid (no ring). There are many other examples. In some ways 2,3,5-triiodobenzoic acid, "TIBA" (XXXIII), behaves similarly both in the pea test and in other growth reactions, but when the concentration ratio between this substance and the auxin becomes large then growth inhibition takes place (12). This was interpreted by Thimann and Bonner in terms of competition between the TIBA and the auxin for the same active centers in the cell. Several workers have found that TIBA promotes flowering and have explained this in terms of its inhibition, at high concentrations, of the action of auxin in the plant. It is evident that the structure of TIBA comes within the grouping of hemi-auxins men-

place in two steps... structures:

$$\text{XXXIII} \qquad n\text{-}C_5H_{11} - CH - n\text{-}C_5H_{11} \qquad \text{XXXIV b}$$
$$\overset{|}{COOH}$$
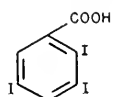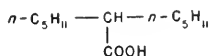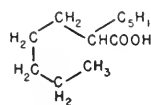
XXXIII   XXXIV a   XXXIV b

tioned above. However, Veldstra, under the term "synergism," describes a similar phenomenon brought about by quite unrelated compounds, especially di-n-amylacetic acid (XXXIV), (14). Although it is true that the related amine, di-n-amylmethylamine, has no synergistic activity, and hence that the carboxyl may be needed, still the relationship between XXIV and the typical auxin structure is somewhat remote.* (If the formula is written as shown at XXXIVb it suggests perhaps a relationship with phenyl-α-propionic acid or auxin-a.) Even if such exceptions should show that Went's generalization is only partly correct, it still seems fair to visualize the enhancement phenomenon as due in some way to the ability of related substances to combine with a given structure.

The Mechanism of the Action of Auxin: A Theory.— It remains only to consider what light all this sheds on the mechanism of auxin action. The discussion of growth as a metabolic process will take place in other papers

*A recent direct comparison, using a sample of XXXIV kindly supplied by Dr. Veldstra, shows that TIBA is the more active of the two.

so only one or two points need be mentioned here. The growth process involves the metabolism of the organic acids. One of the enzymes in this system, which has a sulfhydryl structure, is a critical link: sulfhydryl reagents, such as iodoacetate, inhibit growth strongly and specifically. Malate, pyruvate, succinate, and other acids promote growth; they are used up during growth, and malate or citrate may accumulate when growth is inhibited. Malate and other acids, including malonate, protect growth against the inhibiting influence of iodoacetate. Thus enzymes acting upon the 4-carbon acids play a major role in growth. Viewed in this light the structural requirements for auxin activity take on a striking significance. The protecting action of malonate against inhibition by iodoacetate is only part of a general phenomenon in which substrates and interfering substances or inhibitors compete for the active locations on catalyst molecules, as for example, the relation between O_2 and CO with activated charcoal, or between succinic and malonic or maleic acids with succinic dehydrogenase. In this case malonic acid will protect the enzyme against inactivation by oxidized glutathione. The parallel with succinic dehydrogenase is indeed very close, for Quastel and Wooldridge (7) showed long ago that besides malonic acid and related substances, phenylpropionic acid inhibits this enzyme. Thus the phenyl group can substitute on the enzyme for one of the carboxyl groups of succinic acid. If now we view the auxins as protective substances, we see that the ring takes the place of a carboxyl. The requirement for one carbon atom between the ring and carboxyl means that the auxin is really a malonic acid derivative. Suppose the tissue contains a natural inhibitor of succinic dehydrogenase. (The argument may apply equally to certain other enzymes of the 4-carbon acid series.) For the enzyme to have activity and thus to allow growth it must be protected against this inhibitor; the best protector is indoleacetic acid.

This line of thought at once brings many observations together:

1) Succinic dehydrogenase is inhibited not only by malonic acid but by maleic acid. If auxins are envisaged as malonic acid derivatives, the corresponding maleic acid derivatives should have the same action. These, of course, are the aromatic acids of *cis*-configuration like *cis*-cinnamic acid. If this is true it is the *cis*-structure itself, rather than the angle of the carboxyl, which is important. We should note, too, that the active naphthalene-1-acetic acid and the almost inactive naphthalene-2-acetic acid can be considered as analogous to *cis*- and *trans*-forms.

2) The inhibitors of succinic and of lactic dehydrogenase show marked specificity. Quastel and Wooldridge (7) showed that hydroxy acids, such as hydroxymalonic, which inhibit lactic dehydrogenase, are quite inactive on succinic dehydrogenase. This parallels the marked effect of a side-chain hydroxyl in abolishing auxin activity. The auxin analogue of hydroxymalonic acid is, of course, mandelic acid.

3) The action of malonic acid itself is most suggestive in this connection. In low concentrations (10^{-3}M) it protects against the inhibiting action of iodoacetate on growth. In high concentrations (10^{-2}M and above) it inhibits growth both of coleoptiles and of pea stems. To the plant tissue poisoned with iodoacetate, then, malonic acid acts like an auxin, promoting growth at low concentrations and inhibiting it in high.

4) The 4-carbon acid enzymes, including succinic dehydrogenase, are of critical importance to the life of cells. To protect them against poisoning is essential not only for growth but for life. But the action of a protecting substance implies that it is adsorbed on the enzyme more readily than the inhibitor against which it protects. Hence when the concentration of a protecting substance becomes high its molecules begin to be adsorbed on the enzyme to a degree comparable with that of the substrate; in other words the protecting substance now begins to inhibit. As the concentration increases at first only growth is inhibited, but when inhibition becomes complete the cells are killed. In this way the fact that at high concentrations the auxins become toxic and herbicidal would be explained.

This hypothesis has the advantage of bringing together two hitherto unrelated aspects of the auxin studies, namely the role of auxin in metabolism and the chemistry of the active substances. Perhaps also it may be suggestive to workers in other fields of biology which involve the fascinating relation between structure and activity.

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Growth and Structure of the Primary Wall

THOMAS KERR

T HE primary wall is the membrane surrounding the growing cell showing reversible changes in surface area with variations in cell turgor and permanent changes in surface area as a result of growth. The structure of a membrane capable of undergoing such changes has not been satisfactorily explained.

All cells of higher plants irrespective of their size, shape, or cell-wall thickness have primary walls, and as long as they are in a rapid state of enlargement, the cells have only primary walls. Thus the thickened walls of collenchyma and the outer walls of epidermal cells which are known to be cutinized must be classified as primary membranes. Throughout this discussion the structure of the primary wall will be based chiefly on the structure of a typical parenchyma cell such as one finds in the *Avena* coleoptile.

Chemically the primary walls of higher plants are known to be composed of cellulose and pectic substance. In the cotton hair where thorough investigations have been made, the cellulose of the primary wall gives a typical X-ray diffraction pattern (2), has an average chain length approximately 50 per cent that of the secondary wall (6), and shows standard solubilities of cellulose. Considering the wide variations in chain length in different types of secondary walls it may be concluded that the cellulose molecules of the primary membrane are identical with those of the secondary wall.

For many years investigators working on cell walls have realized that the structure of the primary wall cellulose is fundamentally different from secondary wall cellulose without knowing the basis for the difference. The recent work of Mühlethaler (9) on the structure of the primary wall under the electron microscope shows why these differences

exist. (Fig. 1) Mühlethaler's excellent micrographs demonstrate that the primary membrane is composed of a network of microfibrils in contrast to the more or less parallel arrangement of the fibrillar structure in the secondary wall. The sharply defined microfibrils of the primary membrane are fairly constant in diameter (200–250 Å) and seem to be partly interwoven as in a textile fabric.

Inasmuch as the fibrils of the secondary wall are arranged parallel to each other, fine details of structure can be studied under the ordinary microscope. On the other hand, since the microfibrils of the primary wall are in the form of a network and the fundamental units of the network are below the limits of microscopic visibility, details of primary wall structure under the ordinary microscope cannot be worked out in an entirely satisfactory manner. However, it has been known from studies using crossed nicols, that the microfibrils of the primary wall are not randomly arranged even though they are in the form of a network. It can be assumed that the majority of fibrils composing the network are oriented more or less in one direction. It has been known for years that the cellulose chains in parenchyma cells are oriented chiefly perpendicularly to the major axis of the cell and that this orientation does not change during growth. Striking preparations showing the orientation of the cellulose in the primary wall may be seen when cells are stained deeply in congo red and viewed under a polarizing microscope (Fig. 2). It is well known that congo red accentuates the weak birefringence of cellulose. The stain is prepared as a 0.2 per cent solution of congo red in 1 per cent solution of sodium hydroxide. Pieces of tissue (e.g. whole *Avena* coleoptiles) placed in the dye solution for 48 hours may be macerated easily on a slide by a tap on the coverslip. Thus it is possible to study the orientation of the cellulose in single cells, and even single walls are also easily obtained by allowing a slide prepared in the above manner to dry with a weight on the coverslip. The upper walls of many cells adhere to the coverslip while the lower walls adhere to the slide.

Some years ago, a large number of various primary wall types were studied under the polarizing microscope and from these studies certain generalizations may be made. Whenever a cell possesses an elongated shape, the main orientation of the cellulose fibrils and the major orientation of the primary pit fields are perpendicular to the major cell axis. The regions of the wall in contact with the intercellular spaces, show a second orientation, for here additional fibrils are oriented parallel to

Figure 1. Electron micrograph of the primary wall from a cell of a germinating corn root. Photographed by Mühlethaler.

Figure 2. Primary wall of a parenchyma cell from an *Avena* coleoptile stained in congo red and photographed between crossed nicols.

the major cell axis. The two opposite orientations within the same wall frequently show optical compensation and the parts of the wall adjoining the intercellular spaces may appear isotropic. When one examines a series of cells grading from parenchyma to collenchyma, the second orientation parallel to the cell axis gradually becomes the dominant one. This parallel orientation is the arrangement of the cellulose in the thickened corners of collenchyma. Thus two types of cellulose orientation, one in which the fibrils are perpendicular to the major cell axis, and a second in which the fibrils are parallel to the major cell axis, may be seen in cells that are undergoing rapid enlargement. In neither case is there any appreciable change in the orientation of the microfibrils during growth. Therefore it follows that growth in any direction is independent of the major orientation of the microfibrils within the wall.

The changes that occur in the primary wall during growth have recently been considered by Frey-Wyssling (5). He postulates that the microfibrils of cellulose form a network which is held together by lateral forces, presumably by hydrogen bonding or some equivalent force. During growth the lateral forces holding the fibrils must be loosened, the empty spaces enlarged, and new strands must be interwoven. Frey-Wyssling's hypothesis will explain the plastic extension or growth of the wall. On the other hand, it is extremely difficult considering our present knowledge to see how a wall built in this manner could possess the elastic extensibility of primary membranes. Primary walls are known to expand and contract as much as 30 per cent with turgor changes in the protoplast. Such elastic properties could be explained if the wall were composed of an elastic or rubber-like substance or it could also be explained if the wall were composed of a highly hydrophyllic material that would change in volume and in surface area with the water relations of the protoplast. Cellulose is known to possess rigid molecules having only very limited elasticity and does not display anything of a rubbery nature. Furthermore sheets of cellulose do not change appreciably either in volume or surface area in different neutral solutions with a range of four or five atmospheres. If the wall were composed of a skeletal framework of cellulose, it would be expected to be a nonelastic structure similar to a sheet of cellophane.

The picture is unfortunately more complicated. Properties of cellulose have been evaluated from dried material, and it is now recognized that natural cellulose, that has never been dried, possesses somewhat different

properties. This can be illustrated from studies made on the secondary wall of the cotton hair (3). Cotton fibers of commerce are twisted, ribbon-like structures, whereas the same fibers before drying for the first time are hollow cylinders. During the initial dehydration twists or convolutions first appear and once formed are irreversible. Before the initial drying the cellulose does not give an X-ray diffraction pattern or at the most, only a faint indication of one. This must mean that crystallization or hydrogen bonding of cellulose does not occur at the time of deposition but takes place chiefly during the initial dehydration. The cellulose of the primary walls of cotton hairs likewise gives an X-ray diffraction pattern only after drying. Heyn (7) has reported a similar situation in the parenchyma of *Avena* coleoptiles. Undried cotton fibers show considerable plasticity but when stretched under water, crystallization of the cellulose occurs even without drying. The artificial stretching of primary cells reported by Bonner (4) unquestionably involves not only a reorientation of the cellulose but also crystallization. Dried primary walls do not show the plastic behavior of undried membranes. It is difficult to see how a primary wall composed of a network of microfibrils undergoing a rapid increase in surface area as a result of a sharp increase in turgor, could avoid crystallization of the cellulose. Nevertheless, it is known that crystallization does not take place. Therefore, it is still highly improbable that undried cellulose could form a skeletal network with the elastic extensibility of the primary membrane.

In order to have a better understanding of the primary wall, it is necessary to consider the structure and properties of pectic substances. Our knowledge of the manner in which these substances occur in the wall is still somewhat vague. Preston (11) has recently considered that they are encrusting substances in the same category as lignin. Frey-Wyssling (5) does not mention the pectic substances in his recent discussion of the growth of the primary membrane. Materials in the primary wall grouped under the term "pectic substances" are unquestionably mixtures, including arabans, galactans and possibly other hemicelluloses. However, true pectic substances are apparently restricted to the primary walls and intercellular substance of higher plants and do not seem to be present elsewhere. Pectic substances are now recognized to be composed of straight, long chained molecules of anhydrogalacturonic acid units (8). The carboxyl groups of the polygalacturonic acid may be partly esterified by methyl groups, in which case the substance is called "pectin,"

or partly or completely neutralized by one or more bases as in calcium pectate. The term "protopectin" is applied to the very long chained, parent substance which occurs in primary walls. Protopectin is insoluble in water, but upon restricted hydrolysis gives rise to pectic acid (free of methyl groups) or pectinic acid (partly methylated). The intercellular substance is commonly considered to be calcium pectate.

It is possible to dissolve the cellulose of the primary wall and leave a structural residue of the protopectin. Conversely the pectic substances may be removed and there remains a structural framework of the cellulose. One might expect that long chained molecules of protopectin would be arranged in the primary membrane similar to the long chained molecules of cellulose, but this apparently is not the case. The pectic residue of the wall after the removal of cellulose is isotropic. At no time do the pectic substances within the wall give an X-ray diffraction pattern, and the removal of the pectic substances does not affect the diffraction pattern which is already present. On the other hand, pectins removed from the wall, formed into threads, stretched, and dried, will give an X-ray diffraction pattern (8,10). Furthermore, Owens and his co-workers have given excellent evidence to indicate that pectins possess rigid, rod-like molecules. The length of the protopectin chain is unknown but pectinic acid derived from protopectin by hydrolysis has been reported to have molecular lengths varying from 530 to 1650 Å. (10). Considering all these facts, the arrangement of the protopectin chains within the wall is probably at random or the molecules might possibly be oriented with respect to each other and yet incapable of crystallization.

There are several other properties of pectic substances which are important from the standpoint of their presence in primary walls. 1) Pectic substances are well known for the ease with which they form gels. 2) In calcium pectate, the calcium ion is shared between two carboxyl groups of adjacent chains of pectic acid, resulting in cross linkages. 3) Pectins undergo degradation or depolymerization with extreme ease when in aqueous solutions, and the degradation is markedly influenced by various cations, particularly hydrogen ions.

At least one property of the wall, rigidity, can be associated with the properties of the pectic substances in the wall. Pickles of various kinds from cucumbers, watermelon rinds, or green tomatoes are essentially skeletons of the primary wall preserved in acid, usually after fermentation. During fermentation, cucumber pickles sometimes become soft,

causing considerable financial loss. The softening of pickles is associated with a decrease in the chain length of the pectic molecules (1). Conversely, it is well known that calcium salts harden the texture of pickled vegetables presumably by forming calcium pectate with cross linkages.

Considering all the properties of pectic substances, the property of the primary wall could be explained if it were assumed that the pectic substances were the continuous phase of the wall and the cellulose microfibrils were a discontinuous phase. The cellulose fibrils might be considered as structural reinforcement. Changes that occur during growth would affect the easily hydrolyzable pectic substances and not the cellulose. Changes in elastic extension of the wall could be explained as changes in hydration of the pectic gel. This hypothesis has not been proved but it has been brought forward to get around the impasse that the properties of the primary membrane are not the properties of a sheet of cellulose.

The primary wall has been pictured as a fibrillar network of cellulose. A wall built in this manner would not have the elastic extensibility characteristics of primary membranes. Considering the properties of cellulose and pectic substances, the properties of the primary wall can be explained if it is assumed that the protopectin forms a continuous phase and the cellulose microfibrils form a discontinuous phase.

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Mechanisms of Cell Elongation

HANS BURSTRÖM

General Course of the Cell Elongation

THE mechanism of cell elongation can be viewed from different angles: as a hormone problem, or as a question of morphologic and metabolic changes of the cell. Invited to read a paper on the mechanism of cell elongation, I should like as far as possible to avoid discussing hormones and hormone actions, and concentrate upon the course of the cell elongation. In its general features this problem recently has been awarded an excellent treatise by Frey-Wyssling (8) in a growth symposium paper, but when growth is considered in connection with hormones, the crucial point is not only to find out what happens during the elongation but to find the real inciting cause of the process, where the hormones are likely to exert their action.

Many opinions have been advanced for the cause of the cell elongation, and there is scarcely any part of the metabolism that has not been assumed to form the point of action of auxins and connected with the elongation. Therefore the title, "Mechanisms of cell elongation," obviously covers such a wide subject that I must restrict myself to certain aspects of the problem only.

The superficial cytological course of the elongation has proved to be rather similar in roots and shoots, although these are different with respect to their physiological behavior. Figure 1 shows some well-known main features of cell elongation. The example is taken from the elongation of the epidermis of roots. Growth follows the usual S-shaped curve with a very slow start. The increase in length of the cell is in this case from about 18 to 300μ, and, as is well known, the increase in volume involves largely an absorption of water. Of the osmotic properties the suction pressure of the cell can be determined easily on elongating cells, and this
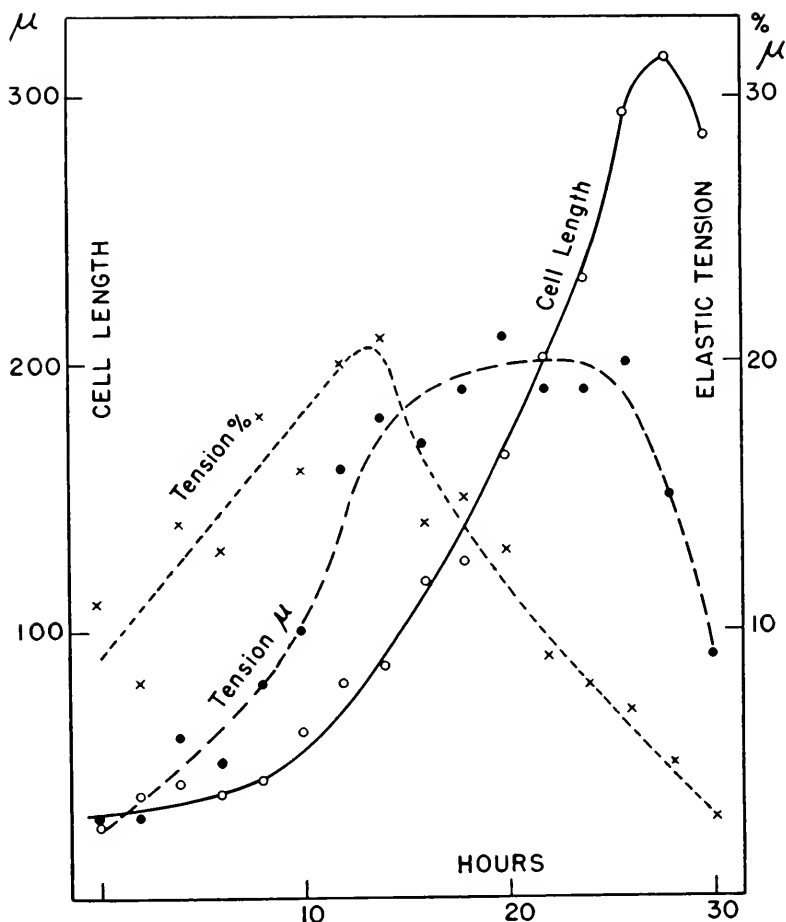
Figure 1. The change in the elastic tension of root epidermis cells during the cell elongation.

has been done both on roots and coleoptiles (2,11). Regarding roots growing in a solution the conditions are simple, the cells are constantly saturated with water during the whole elongation process. In coleoptiles, on the contrary, there is always a large water deficit. In any event it is obvious that the often advanced opinion that elongation must cease in a water-saturated cell is undoubtedly erroneous, and such an opinion cannot even be theoretically sound.

The properties of the cell wall are indubitably of first-rate importance

to the cell elongation. Of the mechanical properties of the wall the reversible, elastic extensibility can be determined with a fair degree of accuracy. Experiences with both roots and coleoptiles have taught that the elasticity always increases at the start of the cell stretching but decreases again before the cells have attained their full size. Figure 1 shows how, in a root, the elastic tension as measured per cell increases rapidly at the start to a maximum value, which is maintained during the main part of the elongation. Relative to the increasing cell length this means a decreasing tension of the cell walls. This increasing elasticity cannot cause the elongation, but it signifies that there are changes in the cell wall at a very early stage of elongation, which undoubtedly are connected with the elongation process.

These observations on the osmotic and wall properties of the cells have led to the assumption that cell elongation proceeds in two phases, which may be rather sharply distinguished from one another and during which different conditions prevail. The first phase involves an increasing elasticity of the wall, which has been explained on the basis of a loosening of the joints between the micellae (7). The second phase is characterized by a hardening of the wall, as evidenced by a decreasing elasticity. During the second phase there is, further, a very rapid supply of nutrients to the cell so that the osmotic concentration does not decrease in spite of the rapid increase in cell volume.

Similar results had earlier been obtained by Ruge (11) with coleoptiles, and he has likewise concluded that the growth proceeds in two steps, the first one involving stretching of the cell without synthesis of new wall materials but with an increasing extensibility of the walls. The consequence is that during this part of the elongation the wall becomes thinner in proportion to the increasing cell surface, and other observations (6,12) have verified that such a spreading of the wall material takes place during the early part of the elongation. During the second phase there is a deposition of new wall material, undoubtedly through intussusception, probably also through apposition. This results in a concomitant hardening of the cell wall. It seems probable that Ruge's two phases for coleoptiles are identical with those shown to exist also in roots, and that consequently shoots and roots behave similarly.

This distinction between the two phases has been further supported by the fact that auxins affect them in different ways (3). The first phase is accelerated so that elongation starts earlier, and the second phase is

shortened, which results in a reduced cell length*and an over-all inhibited growth in roots. These two actions can manifest themselves simultaneously in one root, which supports the idea that there are two different processes going on in the cell elongation.

The first phase above all deserves attention in this connection. We may find there the primary cause of the cell stretching, whereas the ensuing growth by intussusception may be assumed to follow only as a consequence of the preparation made during the first phase.

CELL ELONGATION AND PROTEINS

It is wrong to assume, however, that this is all that takes place during the elongation. Frey-Wyssling (7) has especially emphasized that there is a considerable increase in the amount of cytoplasm during the cell stretching. That this is so in elongating coleoptiles and anther filaments is less significant because they lack a meristem, but the same holds true in roots as shown by Miss Kopp (9). It is correct that the increase up to 90 per cent in volume is caused by water intake, but nevertheless as much as half of the protein synthesis takes place during the cell elongation.

That there is some connection between proteins and elongation is to be seen from the fact that an increase in the supply of nitrogen increases the rate of cell elongation, whereas no such effect is found, for instance, with phosphorus (4). Table 1 shows what happens when excised wheat roots are supplied with increasing amounts of these two nutrients. High concentrations of nitrogen increase the root length. Analyses have verified that an increasing supply of nitrogen leads to increased synthesis of proteins. See Figure 2 for the rate of cell-elongation increases. Note that the time of starting and the duration of the elongation as well as

TABLE 1

Growth of isolated wheat roots with different phosphate and nitrate supplies

SOLUTION	INCREASE IN ROOT LENGTH MM.	CELL LENGTH μ	INCREASE IN CELL NUMBER LONGITUDINALLY
Low P, low N	13.7 ± 1.7	177 ± 3	35 ± 4
High P, low N	25.5 ± 2.0	170 ± 3	120 ± 5
Low P, high N	25.2 ± 1.3	269 ± 4	51 ± 3
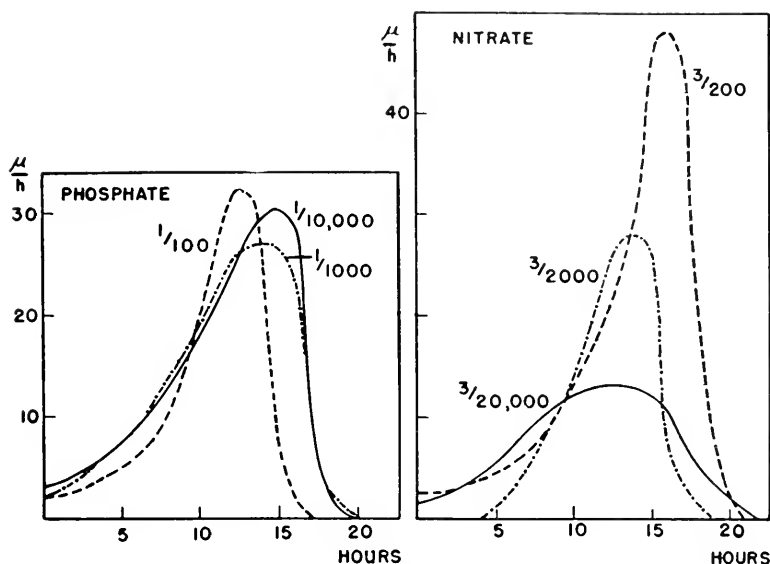
Figure 2. The grand period of cell elongation with different supplies of nitrate and phosphate.

the frequency of cell divisions are unaffected by the nitrate concentrations above the nitrogen starvation level. Only the rate of stretching is affected. Phosphorus, on the contrary, given in concentrations above deficiency levels does not interfere with the cell elongation but only with the cell divisions. It may be that a certain synthesis of proteins is an indispensable prerequisite for cell elongation, because we do not know any instance of cell elongation at present without a protein synthesis. On the contrary, however, there is little possibility that a formation of cytoplasm evokes the elongation. If so, there ought to be a more rapid start or a longer duration of the elongation in response to nitrogen supply and resulting protein synthesis. Further, such a synthesis is a characteristic feature of the meristem, and even though it continues during the elongation it cannot be the cause of the incipient vacuolization and elongation of the cells. Their explanation must be sought in the osmotic properties of the cell or in the conditions of the cell wall.

Elongation and Absorption of Water

The increase in volume depends directly upon an absorption of water, and thus it is necessary to consider under what conditions an uptake of

water may be brought about, with the natural reservation that not every kind of water uptake results in growth. This is also the usual starting point for a discussion of cell elongation, but it is regrettable that there is an obvious uncertainty as to the importance and nature of the osmotic conditions of the cell that regulate the absorption of water. I need hardly remind you of the fact that there is still no unanimously accepted physical explanation of osmotic pressures.

The fundamental formula underlying the water absorption is the one given by Ursprung: $S = O - W$, where S denotes the suction pressure of the entire cell against the external medium, O is the osmotic value or the diffusion pressure deficit of the vacuolar sap including swelling pressures, and W is the elastic tension of the wall, directed towards the protoplast. Unfortunately the properties S and O are often confused.

As the driving force of the growth it is customary to refer to turgor pressure, or the pressure acting from within the cell as a force striving to expand it. What turgor signifies can be theoretically deduced and expressed by the formula $T = O - E$, where T denotes the turgor pressure, and E the osmotic value or diffusion pressure deficit of the solution surrounding the cell, that is, the external solution. The turgor pressure can thus be defined as the difference in diffusion pressure of the water outside and inside the cell, or the force with which water tries to enter into and expand the cell (5).

These two formulae are, as a matter of fact, quite independent of each other. They contain three independent variables: O, E, and W, the last of which is in reality not an osmotic property in the strict sense, but is, as already mentioned, the elastic wall tension.

The equations further signify that there are two existing forces or pressures in the cell and not more, namely W, the elastic pressure of the wall, directed inwards, and T, the diffusion pressure of water, i.e., the net diffusion pressure, which normally as in turgid and expanding cells causes a pressure directed outward toward the wall but in shrinking cells is directed inward. These two pressures are in principle quite independent of each other. If they are equally large, that is, if $T = W$, the cell is in equilibrium with its external medium. Then also $S = E$, which is the usual way of expressing that the suctions of the cell and of the external medium are equal. We must assume that before elongation starts the cell is in such an equilibrium with its surroundings. The rela-

tion between turgor and wall pressure is illustrated in the osmotic diagram in Figure 3.

In order for absorption of water to occur it is necessary that T exceed W. We may therefore say that the pressure from the inside causes an expansion of the cell; but from the equations it follows that in such a case S must also exceed E to the same extent, so we may quite correctly also say that the suction of the cell is greater than that of the external medium. Hence water is absorbed and the cell expands. Expansion is caused by a difference, $T - W$, or in other words, $S - E$; it may be called a turgor-over-pressure or water deficit of the cell. It is thus immaterial if we call the resulting increase in volume an absorption of water owing to a water deficit or an expansion owing to a turgor-over-pressure.
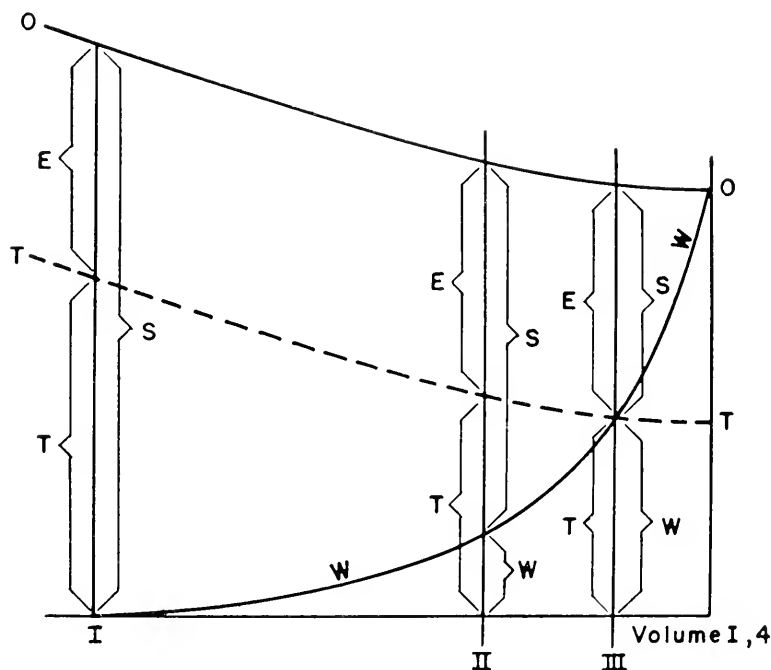
Figure 3. Diagram showing the osmotic conditions of one cell at equilibrium or nonequilibrium with one external medium. O ————— O osmotic value of the cell sap, T ———— T turgor pressure, W ———— W wall pressure, E osmotic value of the external medium, S suction of the entire cell. I = incipient plasmolysis, II = the cell absorbing water, III = equilibrium with the medium E.

Note, however, that when the cell is at equilibrium, the turgor pressure is balanced by the wall pressure and therefore cannot cause any expansion of the cell, because water cannot be absorbed without a suction difference.

These relations are still under discussion, but for the given reasons I am inclined to believe that the computations recently made by Frey-Wyssling (7,8) of the osmotic work performed in the elongation of cells are not correct. He has assumed the cell to be expanded by a force $= W$, whereas in reality it should have been T, and these two values need not be equal, certainly not during water saturation, and perhaps not during the elongation.

This connection between turgor and water absorption is not generally realized. Audus (1), in his monograph on auxin actions, brings out Heyn's opinion that the turgor pressure can call forth a stretching of the cell, which ultimately implies a reduced hydrostatic pressure of the cell contents, the consequences of which are an increased suction force and water uptake in active cell stretching. Such a proposed sequence of events has no purpose. The conditions are much simpler, for "stretching under a turgor pressure" is but another way of saying "water absorption owing to a suction difference."

Looking for the cause of such a water uptake we must find out what can cause a change from the equilibrium conditions when $T = W$ and $S = E$. This is possible by changing one of the three variables of the equations, E, O, or W. E can, of course, be omitted from the discussion leaving O and W to be considered.

An increase in O implies an increase in the amount of osmotic material in the cell. That an actual increase in nutrients occurs from the start of the elongation is easily demonstrated. The result should be an increased turgor pressure, $O - E - W$, causing absorption of water, a rise in the wall pressure, and a decreasing turgor until a new equilibrium is attained, with O, W, and T all higher than before. In practice there is a decrease in O during the most rapid elongation, so that such a process alone cannot be responsible for the cell elongation, Nevertheless it is necessary to consider to what extent absorption of water in itself can contribute to the cell elongation. Especially so, because such mechanisms and even an auxin-induced water uptake without any change in the osmotic conditions proper play a certain role in the discussion of the cell-elongation process.

The most elaborate theory to that effect has recently been advanced by Pohl (10). By studies on plasmolysis and deplasmolysis of coleoptiles he has verified in a highly convincing way the point that auxins increase the permeability of the cytoplasm to water. He has concluded that the cause of the elongation is an auxin-induced rise in the water permeability, causing a water uptake and an increase in volume of the cell. There is a difficulty in this interpretation, however. It presupposes that the increase in volume of the cell is limited by the rate of water absorption. Now it is generally assumed, for good reasons, that the water permeability of the cytoplasm is very high and that it can scarcely hamper the water uptake if the osmotic conditions permit an absorption of water. Of course the rate may be affected but not the end result. Pohl has therefore been compelled to assume that initially the tonoplasts are practically impermeable to water. This is a highly hypothetical assumption which, of course, ought to be verified cytologically.

The immediate consequence of this or any water absorption is a reversible elastic extension of the wall along the curve *W* of Figure 3, and it need not be emphasized that this is different from the elongation which occurs in growth. The problem is, however, whether such an elastic extension may change into an irreversible, plastic deformation.

This concept of plasticity in the physical meaning applied to living cell walls has been repeatedly criticized with regard to the special micellar structure of the wall and the extreme and unnatural experimental conditions necessary for a demonstration of such a passive, irreversible stretching of the cell wall. Not even Pohl was able to explain the elongation by means of a water absorption alone, but he had to round off his theory by assuming an additional change in the cell wall, without which cell stretching is precluded.

The reason Pohl has found it impossible to explain elongation and auxin action by a change in the wall and the wall pressure only is his emphatic postulation that the suction pressure of the coleoptiles amounts to 6 to 7 atmospheres and the wall pressure to only 1 to 2 atmospheres. Thus any change in the wall pressure must be of relatively little importance and cannot explain the rapid elongation following an auxin treatment. This deduction is wrong, however. Before growth in length starts the wall pressure is only some few atmospheres whereas the cell is probably in equilibrium and the turgor is as low as the wall pressure. There exists no turgor-over-pressure or suction deficit with respect to

the surroundings. Every decrease in W below T may thus be of relatively great importance even if its absolute amount is small.

In fact, when the water deficit of a cell is large and the wall pressure is low a decrease of the latter permits a relatively much greater absorption of water and an increase in volume of the cell than when the cell is more nearly water saturated. That follows from the shape of the W-curve in Figure 3. To obtain the same increase in volume only by a water absorption without active changes of the wall, enormous forces of water absorption would be required, irrespective of whether they were of osmotic or so-called nonosmotic origin.

There remain to be considered inevitable changes of the wall pressure as a starting point of the elongation. It is hardly necessary to emphasize the fact that if there is an active change in the wall structure, it is futile to speak of plastic extension as the cause of the elongation because this presupposes only a passive stretching under the influence of an external load.

Elongation and Changes in the Cell Wall

The next step is to find out what direct evidence there is of active changes within the cell wall. One fact seems to be firmly established—that the walls of growing cells always have a tubular structure (8). This means that the micellae are generally orientated in a transverse direction, neither the stretching during the first phase of the elongation nor the intussusception seems to alter this structure of the wall.

As already mentioned it is probable that the growth during the first phase involves an increase in surface of the cell wall partly at the expense of its thickness. This means that wall material is translocated within the wall itself, and the increase in surface depends upon a kind of growth by intussusception, even though the material deposited in the wall is not delivered from without. If this were a mere plastic remodeling of the wall substances this fact would become apparent from changes in elastic properties of the wall which are most conspicuous signs of something happening in the wall at the start of the elongation.

To take some figures from one series of measurements on roots, the cell length increases from 20 to about 400μ. The elastic stretching under a constant turgor pressure increases, as already mentioned, from about 3 to a maximum of about 40μ per cell when the cell reaches a length of about 120μ. From then on the elastic stretching remains constant.

The specific tension of the wall showing its elastic properties can be computed in different ways. The tension must of course be related to the cell length, but attention must also be paid to the thickness of the wall. The theoretical formula reads

$$dl = \frac{l(1 - n)P}{2Ed},$$

where dl is the elasticity, l the cell length, d the wall thickness, and the rest are constants. In applying this formula to my material Frey-Wyssling (7) has assumed the wall thickness to be constant and has constructed a graph on this assumption (Fig. 4). The graph gives the modulus of elasticity which runs inversely to the tensibility of the wall. During the first phase of the elongation this computation gives a tenfold increase in the elasticity of the wall.
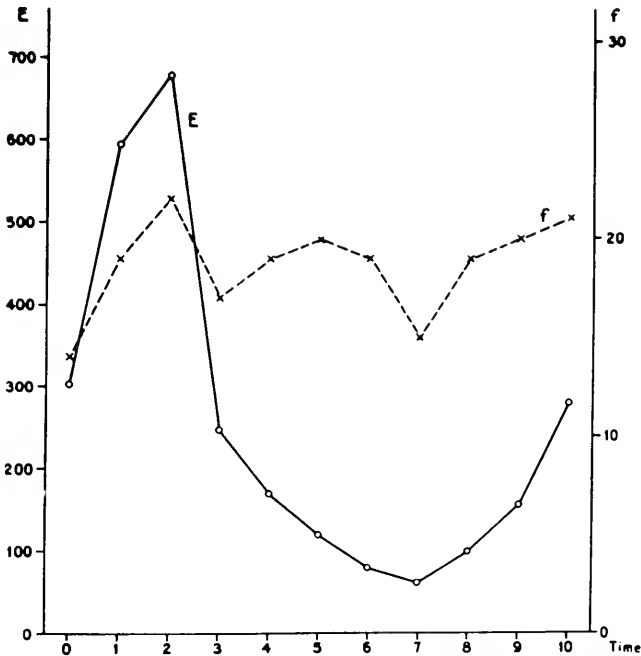
Figure 4. The elastic properties of the walls of elongating epidermis cells. Both graphs deduced from one series of measurements (2). E the modulus of elasticity according to Frey-Wyssling, f the rigidity of the wall according to the formulae of Tamiya.

As a matter of fact we do not know whether the wall thickness remains constant. It is possible that it decreases during this first phase, which would cause an increasing tension in the wall even if its structure remains unchanged. On such an assumption the real increase in the elasticity should not amount to more than a doubling. These are minimum figures and those of Frey-Wyssling are maximum values for the loosening of the wall structure.

There cannot be merely a loosening of the wall structure, however. Assuming a constant amount of wall material and taking into account the fact that the cell wall maintains its micellar structure during the whole elongation process, there must be some factor responsible for this organized translocation of material within the wall. Assuming with Frey-Wyssling that the wall maintains its thickness from the start of the elongation, it follows that there must be from the very onset a considerable formation of new wall material. In neither case is there reason for believing that the initial phase of the elongation is only a loosening of the wall structure. Although such a change occurs and plays a prominent role, we must always also consider some mechanism establishing the fixed structure of the wall.

All these computations have been carried out under the assumption of a proportionality between pressure and tension of the wall, but this is erroneous as is shown by Figure 3. Tamiya (13) has deduced formulae of wall pressure and suction in relation to turgor tension which include a factor for the rigidity of the cell wall. The formula reads:

$$\text{Tension} = \frac{fC_i}{fC_i - 1 + e^{-fC_i}}$$

where C_i denotes the osmotic value and f the rigidity of the wall. If his formulae are applied to elongating cells it is found that the rigidity factor f is remarkably constant in spite of the very large variations in the elasticity as computed according to the ideal physical formulae. This is shown by Figure 4. The cause of this disagreement is that in this case the cell walls are assumed to obey ideal laws of mechanics, whereas the rigidity is computed with attention paid to the empiric formulae of the osmotic conditions (13). The two graphs of Figure 4 are constructed from one series of measurements.

Obviously there are several ways of computing the elastic properties of the wall, and different results can be obtained. We have certainly

too incomplete a knowledge of the physical properties of the cell wall to be entitled to draw any definite conclusions, but it seems probable that even if some changes occur in the wall properties, nevertheless during the elongation these properties remain remarkably constant.

Another complication in the evaluation of all data on the mechanical properties of the cell walls is the fact that in several instances the elongation does not take place uniformly over the whole cell surface. Apical growth is a well-known feature of many cells, and the differential growth reported by Sinnott is probably a widespread phenomenon in ordinary tissues. The consequence is that the walls of growing cells are also heterogeneous with respect to their mechanical properties. In the case of root-hair cells the basal parts may be elongated on an average 9 per cent under a given turgor pressure whereas the apical parts will be changed as much as 25 per cent. This shows that the two ends of one growing cell must have different structure. Thus, average figures for whole cells do not show the properties of the restricted areas where growth takes place at any given moment.

The conclusion to be drawn at present from available data is, however, that we cannot abandon the old idea that the fundamental principle of cell elongation is an organized, active growth of the wall. It is also obvious, however, that the experimental data concerning the cell-wall properties are too meager to permit more than a vague hypothesis of what takes place during the cell stretching. Nevertheless, any theory of the cell elongation and hormone action in growth must take into account the active formation of the wall.

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Control of Evolution and Life Processes in Plants

ALBERT F. BLAKESLEE

As a college student I was often driven to employ a not uncommon technique of asking questions when I was unprepared to recite. This must have had a measure of success since one of my professors who was unable to say anything more favorable in my behalf, wrote as a recommendation that I asked intelligent questions. I feel impelled to use the same technique here since I feel woefully unprepared to contribute much in the way of explanation of the mechanisms of chemical control of cell division in plants—the topic originally assigned me for this symposium. I can, however, think of many questions I should like to have answered regarding these important processes.

A question is the basis of every research. To be fruitful, however, a question need not be intelligent, if by intelligent is meant approved by conventional judgments. Such conventional questions will often be found to have been already answered. It is the unusual that most easily arouses our curiosity. For this reason botanical gardens generally feature exotic plants and not the native flora. The commonplace—what is continually happening under our observation or continually not happening—although more fundamental than rare occurrences, is usually considered as an axiom and not as a matter for inquiry. Unconventional questions and those which would generally be called foolish may be the most rewarding. Laymen who are unhampered by too great familiarity with one's field of research and stereotyped ways of thinking often ask the most penetrating and suggestive questions though they generally apologize for asking what they think may be a foolish question when they know so little about the subject. It is one of these foolish questions which has been asked me on several independent occasions that I wish to pass on to you as an introduction to my discussion of the control of

evolution and life processes. The question has each time been conceived as a jest and yet it touches at the very heart of our understanding, or rather lack of understanding, of the processes of sex and reproduction. If when we gather here again to celebrate the 150th anniversary of the natal day of the University of Wisconsin, we have a complete answer to this question we can feel that the intervening 50 years of research have been well spent. The challenging question which has been asked of me and which I am passing on to this symposium is—why can't you cross corn and beans and get succotash? I confess I don't know the answer. Does anyone in the audience?

We will return more specifically to the subject of crossability later on. It might be pointed out now that the question mentioned implies the desirability of translating knowledge through action toward a directed goal. For a long time the various aspects of biology tended to be studied as separate entities. The structure and behavior of chromosomes for example were of interest to cytologists without relation to the transmission of hereditary traits, and some leading geneticists seemed to resent the use of chromosomes in interpreting genetic behavior. This stage is passing and cytology and genetics have profitably joined, with evident hybrid vigor, to form cytogenetics. Structures and processes are being studied in order to interrelate and understand what the organism is and does. A still newer phase of research is under development—the utilization of this understanding of all the aspects of the plant in consciously controlling its life processes and directing its evolution. It is about this phase of research, which we may call that of the genetics engineer, that I wish to speak a bit. I shall use largely the genus *Datura* for examples to show how new knowledge can be put to work in interpreting biological behavior and in molding form and function. This is because I am personally more familiar with this plant than with most other plants, but especially because I am familiar with the important research of associates who have worked with *Datura*. Though I have had something to do with raising certain of the problems it is to these others that we are generally indebted for their solution. In acting as spokesman for the *Datura* workers, therefore, I feel as if I were shining by reflected light.

Because of its ease of handling *Datura* has been much used by the early hybridizers. With it Naudin discovered segregation in the F_2 generation before Mendel's classical paper on inheritance in garden peas.

He thought he had discovered visual evidence for the separation of maternal and paternal essences in the F_1 generation since in a hybrid between a form with spiny capsules and one with smooth capsules he sometimes found capsules one half of which was smooth and the other half spiny. This is an example of a correct conclusion from faulty interpretation, since more than 50 years later we were able to show that Naudin's findings were due to a virus disease which can be propagated by grafting. This environmentally induced type we called "Quercina" from its lyrate leaves. Almost identical effects are produced by a recessive gene which arose from X-ray treatment. DeVries used the segregation of two pairs of characters in *Datura* in rediscovering the laws of Mendel.

Of modern students of *Datura* I want first of all to speak of John Belling, a brilliant pioneer in cytogenetics. His finding that certain mutant types in *Datura* had single extra chromosomes, which in many cases he could distinguish by size and structure, not only furnished an interpretation of our trisomic ratios from Primary $2n + 1$ types but showed that such types were due to the unbalancing effect of specific chromosomes. This was at the time when some of the leading students of *Oenothera* believed that extra chromosomes were the effect and not the cause of $2n + 1$ types. It was nearly 20 years after Belling's findings before the successor of de Vries publicly acknowledged that extra chromosomes were the cause of mutant types in the Evening Primrose.

In another group of $2n + 1$ types which we now call Secondaries, Belling found that the extra chromosome was often associated with two others at metaphase to form a closed trivalent. This could only mean, he believed, that the extra chromosome of the trivalent must be a doubled half chromosome. He was the first to put effectively to work the idea that at metaphase similar chromosome ends are attached like dominos. This interpretation not only explained the morphological peculiarities and breeding behavior of Secondaries but laid the way for the later discovery of segmental interchange.

In 1921 there appeared in our cultures a compensating chromosomal type "Nubbin" among the offspring of a plant treated by Gager with radium emanation. This is probably the first induced chromosomal mutation. A couple of gene mutations in the selfed offspring of only 18 parents also were found from the same treatment but their number was too small to warrant being sure that their appearance was due to the radiation. Later Muller from his brilliant work with *Drosophila* gave

ample evidence that genes may be caused to mutate by radiation treatment. Ratios of purple to white flower color in the offspring of our induced type Nubbin showed it to be a convenient form with which to separate the A and B chromosomal types in races from nature which we had earlier distinguished by tedious breeding procedure and by the fact that B races occasionally threw certain abnormal $2n + 1$ types. It was in one such type that Belling found two large chromosomes attached to a small chromosome to form a trivalent at metaphase. The fact that like chromosomal ends become attached, which he had earlier used in interpretation of Secondary $2n + 1$ types, he again put to work in explaining our B races. He concluded that two of their chromosomes could have been derived from the A race by interchange of segments of two nonhomologous chromosomes. As shown by Bergner and Satin, segmental interchange has been responsible for the formation of so-called Prime Types in all the species of *Datura* of which an adequate number of races have been investigated. It has accompanied the formation of species in this genus. The theory of segmental interchange laid the basis for interpretation of the peculiar genetic and cytological behavior of the *Oenotheras* which had baffled students of heredity since de Vries first published on their mutability.

Through the utilization of Secondary chromosomes and Tertiary chromosomes which had been formed by segmental interchange, it was possible to synthesize pure-breeding types with predicted characteristics. As examples the following types all with similar characters due to the extra 2 half chromosome may be mentioned: $2n - (11 \cdot 12)_2 + (2 \cdot 11 \cdot 12)_2$; $2n - (1 \cdot 2)_2 + (\cdot 1)_2 + (2 \cdot 2)_2$; $2n - (13 \cdot 14)_2 - (23 \cdot 24)_2 + (2 \cdot 14)_2 + (13 \cdot 23)_2 + (\cdot 24)_2$. It has been pointed out that such types should not be called synthesized new species since they possess no isolating mechanism which would prevent them from crossing with the normal type. However, if their chromosome number is doubled (and this has been done through treatment with colchicine) such crossing is almost completely prevented.

Since tetraploids are a source of triploids and thus of the full range of the Primary $2n + 1$ types, methods have been sought for doubling the chromosome number. In 1937 we stumbled on the use of colchicine for this purpose. Colchicine apparently prevents the process of spindle formation without interfering with chromosome division. Hence the chromosomes may divide several times before the effect of the drug

wears off and spindle fiber formation and cell division is resumed. It should be pointed out that a pure $4n$ branch is probably never produced as the immediate effect of treatment with colchicine or other polyploidizing agents. Dr. Bergner has shown that individual chromosomes are dropped out either in connection with the doubling or without any doubling of the chromosome number. Colchicine, therefore, is a method of securing $2n - 1$ plants. Furthermore it is only the cells which are in division which have their chromosome number doubled. The resting cells are not affected. The first effect of the treatment is a rough-leaved mixochimera branch in which some of the cells are $4n$ and others normal $2n$. Out of such a mixochimera may grow a smooth-leaved branch which may have $2n$ or $4n$ cells. As Dr. Satin has shown, such a branch may be a periclinal chimera with different numbers of chromosomes in each of the three germ layers. She has put this fact to work by labeling the three germ layers with different polyploid chromosome numbers and in this way has been able to determine the contribution which each germ layer makes to the organs of the adult plant. She has thus been able to show that the classical interpretation of the stamen as a modified leaf is incorrect. From the fact which she discovered, that the transmitting tissue in the style through which the pollen tubes grow is of epidermal origin, we have been able by using a periclinal chimera with a $4n$ outer layer to get abundant crosses between a $2n$ female and a $4n$ male. Such a cross would not usually be possible between normal $2n \times 4n$ parents since the pollen tubes from the $4n$ parent, as Buchholz has shown, burst in the $2n$ tissue of the female style. Dr. Satin's findings have been extended by others to spontaneous bud sports of fruit trees which investigation has shown are often periclinal chimeras.

Autotetraploidy in which each kind of chromosome is represented 4 times is of interest to floriculturalists since it usually causes an increase in flower size. It has also occurred in nature. The greatest interest in methods of doubling chromosome number lies in the ability which it affords of producing new fertile and pure-breeding forms with hybrid vigor from sterile "mule" plants. This is known to have been a method of evolution in nature and has given us some of our best economic varieties in wheat, oats, tobacco, cotton, and timothy grass. In the development of these forms we have had to wait for the chance hybridization between distantly related species and the rare doubling of the chromosomes of the sterile hybrid. Now by the techniques of

embryo culture we should be able greatly to increase the number of wide hybrids between species, and by the use of colchicine we should be able to transform many of them into pure-breeding double diploids or new species.

Having found a method for doubling the chromosome number it seemed desirable to find a method for halving the number. Such a method would have considerable theoretical and economic importance. A plant breeder, for example, may wish to combine in a single variety of wheat the resistance to different rusts and smuts, high yield, good milling, and other desirable qualities by crossing two varieties together. The resulting hybrid will be highly heterozygous and it may take several generations and much labor before he is able to secure a pure-breeding type with the combination of characters he desires. If he could only induce the reduced egg cells to develop without fertilization he could get homozygous individuals in two jumps since the $1n$ plants from unfertilized eggs can be readily induced to double their chromosome number— at least such is the case in *Datura*. We confess we have not yet succeeded in our attempts to induce unfertilized eggs to develop but we believe the problem is soluble since the plant does it as shown by the fact that we have found over 200 spontaneous $1n$ plants in our cultures of *Datura stramonium*. A related question is what are the factors which determine whether a cell shall divide mitotically retaining the $2n$ condition in the daughter cells or divide meiotically thus halving the chromosome number. Perhaps work now under way in the University of Wisconsin will help us to the answer. Through the use of auxins one has a measure of control over the production of roots. It would be of convenience to the plant breeder if he were able similarly to induce shoot production. (This sentence was written before I attended the meeting of the Growth Society in New London where I learned of the work of Skoog in controlling bud and root formation through interaction of adenine and auxin.) The plant breeder's next request would probably be for a method of inducing flower formation or usable reduction divisions wherever and whenever he wanted them.

We now return to the question of our introduction: Why can't one cross corn and beans and get succotash? This naturally leads us to consideration of the barriers to crossability between species. We need not mention at this time the various ways in which pollination is hindered or prevented such as geographical separation, or flowering at different

times. There are a series of problems the plants have to solve or barriers that have to be overcome before a hybrid between species is possible, assuming pollination has taken place. The first problem is that of germination on the stigma of the female parent. Pollen germination may be induced by manipulation of the osmotic pressure and the chemical constituents of artificial media. The function of a pollen grain is by means of the pollen tube to carry the two sperm cells down the style and discharge them into the ovule. As Buchholz had shown, in many species' combinations the pollen tubes burst in the foreign style and are put out of commission before they reach the ovary. In one case we were able to overcome this bursting. One of our students, Miss Carmen Sanz, found that most of the tubes burst when pollen of tomato was used on diploid styles of *Datura stramonium* but that most of them grew without bursting when used on tetraploid styles. Some of our students have had a measure of success in getting slow-growing pollen tubes down to the ovary by shortening and splicing the styles. By these two methods, therefore, the barriers of slow-growing and bursting pollen tubes have been partially overcome. It is surprising how many species are able to overcome the earliest barriers to crossability. Miss Sanz tested the pollen of over 60 species on the pistil of *Datura stramonium*. She found that in nearly half the cases the pollen germinated. It was especially surprising that several of the monocots such as Freesia and Tulip showed relatively good germination though their pollen tubes grew only a short distance in the *stramonium* styles. What is it that slows up the growth of pollen tubes in such wide pollinations or causes their tubes to burst? Perhaps the difficulty is related to the cause of bursting of tubes from a $4n$ parent in the $2n$ style of a female, the cause of which is still unexplained.

A special study has been made of crossability in the 90 combinations among the 10 species of *Datura*. We had anticipated that failure of egg and sperm to unite might be an important barrier to crossability. There is no evidence that this is the case in *Datura*. Apparently whenever the egg and sperm are brought into contact they fuse. Barriers come later which may prevent their further development. In some hybrid combinations no division of the fusion cell takes place. In other combinations cessation of growth occurs after 6 or 8 cells at most are formed. In still other combinations young hybrid embryos of various stages of development are produced. In only 19 species' combinations were viable seeds

formed, and two of these produced a single good seed only after several hundred pollinations. In some cases, especially when long-styled species are pollinated by short-styled species, the barrier to crossability may be due to inability of pollen tubes to reach the ovary before the style abscises, because of their slow growth or early bursting. The major barrier to crossability in our group, however, is the cessation of development of the hybrid embryos. What stops the growth of the embryo? For this we have some evidence.

In selfs and compatible crosses, Dr. Satin has found that the cells of the integument are filled with starch grains at early stages of development. Apparently the endothelium, a single layer of cells immediately surrounding the embryo sac, functions as nurse cells and through its activities the cells of the endosperm become filled with fat and granules of aleurone which are ultimately digested and passed on to the normally developing embryo. The endothelium itself also becomes digested. In incompatible crosses, however, the picture is quite different. The number of starch cells of the integuments increases and the endothelium, instead of remaining a single layer of cells, proliferates and may invade the embryo sac and form a tumorous tissue. In consequence, apparently, the embryo aborts. This abortion is evidently not due to any lack of food but rather to inability of the embryo for some reason to utilize the abundant food present or to some factor responsible for the inhibition or digestion of the embryo.

Rappaport has given further information regarding the causes of embryo abortion. He finds that there is a water-soluble, thermostable inhibitor which can be extracted from the disintegrating material which surrounds the aborting or arrested hybrid embryos. This inhibitor stops the growth of normal selfed embryos both *in vitro* and *in vivo*. Normal ovules which have been arrested by injection of a solution of the inhibitor furnish an extract which inhibits embryo growth when injected into another young ovary which again develops an extractable inhibitor.

Why wide crosses between certain species cause the production of ovular tumors and embryo inhibitors is not clear. It cannot be due to incompatibility of different kinds of genes from the two parents since the same type of ovular tumors with embryo abortion had been earlier found in the cross $4n \times 2n$ and $2n \times 4n$ when both the diploid and tetraploid parents were in the same highly inbred line. Moreover a similar production of ovular tumors is obtained, as shown by van Over-

beek and Conklin, when an auxin such as naphthaleneacetic acid is injected into young castrated ovaries. It appears that the endothelial layer has a lower sensitivity threshold to certain chemicals than the other tissues of the ovule. We are attempting to induce the endothelial tumors to grow in tissue cultures but so far without success. Why certain tissues are easily cultivated and others are difficult is not clear. Also we do not know why a fertilized $2n$ egg in a diploid ovary develops readily to a mature seed but an unfertilized $2n$ egg in a tetraploid ovary usually fails to develop. The obvious objective in our endothelium problem is to find an anti-inhibitor which will neutralize or prevent the activity of the embryo tumors.

Now how can these facts be put to work? Drs. van Overbeek and Marie Conklin several years ago developed special methods for the culture of excised normal embryos of *Datura stramonium* on artificial media. It was only the larger embryos which could thus be induced to develop further under the conditions used. The fact that sometimes haploid ($1n$) seedlings have come from twin embryos within the same seed coats suggested that there might be some stimulating substance in the normal embryo sac which induced the development of the twinned $1n$ embryo. Following this suggestion, coconut milk, which is a natural endosperm, was tested and found to be an effective embryo stimulator which incited growth in excised embryos as small as 0.1 mm. in diameter. Later, on coming to Smith College, we used the embryo culture technique to secure hybrids in wide species crosses. We have found malt extract more convenient than coconut milk as an embryo stimulator. Since when autoclaved it develops toxic substances, however, we have had to sterilize it with a Seitz filter.

The embryo technique we have put to work in getting hybrids with which to determine the interchanges which have taken place in the evolution of our ten species of *Datura*. It also has given us information regarding important barriers to crossability. We have already said that of the 90 species' combinations only 19 gave viable seeds capable of germinating. In addition we have secured dissectable embryos from 45 species' combinations. Of these 31 have been grown to a stage of maturity at which it could be certain they were the result of hybridization. The others may have been hybrids, but it sometimes happens that the dissected embryo turns out to be a haploid ($1n$) derived from the development of an unfertilized egg, and sometimes an embryo is due to

a slip in technique and not to the cross intended. In 5 cases sectioned material showed that fertilization had occurred but growth had not proceeded far enough to be detected with a dissecting microscope. There were 21 cases which have been recorded as negative, presumably because the pollen tubes were not able to reach the ovary.

Embryo culture of hybrids of our 10 species of *Datura* raises many problems. It has taught us to think of growth and development as determined by the internal microenvironment through the interaction and balance of chemical stimulators and inhibitors. Even now with our limited knowledge and inadequate techniques the possibilities of greatly increasing the number of wide species' hybrids, some of which may be transformed into pure-breeding new species, is certainly alluring. With further knowledge and improved techniques more of the barriers to crossability may be found to be removable, and plant breeders may inaugurate an age of massive miscegenation. It may then be possible to answer the question of our introduction and to say that we can cross corn and beans. It will be interesting to see what the offspring will be from all these crossings.

In closing I wish again to apologize for speaking only of work on *Datura*. Other investigators might make similar statements about the plants with which they have been working. With examples from studies of the plant which I know best I have tried to emphasize the desirability of learning all we can regarding the intimate structure and behavior of the ultimate elements of the plant, but also of interrelating this knowledge toward a better understanding of the whole life of the plant and especially toward the utilization of this knowledge in the conscious control of its life processes and evolution.

Knowledge is indeed power—potential power—but knowledge which is not put to work is sterile.

Twenty Years of Plant Hormone Research

F. W. WENT

IN TALKING on the history of plant hormones, I want to trespass first on the era before definite information on the subject was available. It is most illuminating to see how many excellent starts were made experimentally and theoretically before the overwhelming pressure of evidence broke through the inertia of plant science as a whole, and plant hormones became generally accepted as important factors in plant development. I still remember an afternoon in 1933 when I was discussing plant growth with some students and assistants of one of our big universities. Inadvertently I used the words "plant hormone" which immediately illicited the condescending question, "Do you really believe in plant hormones?"

In and before the eighteenth century the biological sciences were still predominantly descriptive, with only here and there some remarkable inroads by the experimental method, such as were made by Stephen Hale and Jan Ingenhousz. When we find here the first beginnings of the hormone concept, it is merely descriptive without any vestige of experimental evidence. Thus Duhamel Du Monceau makes descending sap responsible for root formation, and Agricola is even slightly more specific in that he talks about a "materia," or substance which causes root formation.

Then in the nineteenth century, after a period of contemplation or *Naturphilosophie*, botany unfolded into an experimental science. It was largely the genius and untiring work of Julius Sachs which led to the creation of a picture of the life processes in the plant.

These first years of plant physiology were largely given to analysis, in which many processes inside the plant were studied separately without trying to find links between them. There was so much to be done before

a synthesis could be attempted. Yet a number of investigators, among whom Sachs, Beijerinck, and Darwin should be mentioned, tried to view the plant not only as an agglomeration of diverse reactions but as an organism. Thus they came to an appreciation of interrelationships between different parts. Darwin (7), for example, found that in grass seedlings and in roots the response to light and gravity is produced by a bending of the zones several millimeters below the tip of the organ, yet that the tip itself is essential to the execution of the curvature. His clear mind saw immediately the implications of this behavior, namely, that some sort of connection between tip and responding region of the organ existed. To make this behavior clear to his readers, he drew some parallels with animal behavior, which caused a storm of protests. Thus the essence of the phenomenon itself was overlooked by fighting about its wording. Certainly his tentative suggestion that a substance brought about this correlation was never taken seriously.

Whereas Darwin came upon the idea of links between different parts of a plant through a study of tropisms, Sachs (18,19,20,21) hit upon the existence of chemical messengers through his studies of the flowering behavior of begonias and squashes, and the rooting responses of cuttings. He clearly showed that in addition to ordinary nutrition, which makes nongreen parts dependent upon leaves, more specific responses were evoked by leaves, and through clear reasoning he came to the conclusion that minute amounts of chemicals, moving polarly through the plant, were responsible for differentiation of roots and flower parts.

Beijerinck (2) came to a similar conclusion based on entirely different facts. He had observed that galls were produced by extremely minute quantities of substances given off by the developing larvae of gall insects, or in one specific instance by the mother insect while it laid its egg. He coined the name "growth enzymes" for such substances, because their effect did not seem to depend on their quantity but rather their quality. Both Sachs and Beijerinck made some abortive experiments to extract their correlation carriers or growth enzymes. But these arguments were sufficient by themselves to indicate their existence, according to our present-day views. In those days, more than half a century ago, their arguments were not considered valid, and even in the present century much experimental effort was expended in the disproval of Darwin's, Sachs', and Beijerinck's views.

It should be stressed that all these considerations, showing how essential the idea of chemical messengers was for the interpretation of phenomena observed in plants, were published 10–25 years before the hormone concept was introduced in animal physiology. If the other botanists of that time had been more constructive in their thinking and less of the flaw-picking variety, plant hormones might have been a reality long before the more tedious proofs for the existence of hormones in animals had been produced. However, there should be no crying over spilt milk; we should try to learn by the achievements and the faults of our predecessors.

After this first brilliant period in which from so many different angles evidence for the existence of correlation carriers in plants was adduced, and after the first reaction to this in the form of negations, a period of consolidation started. Many investigators (Haberlandt, Ricca, Errera), among whom I should like to single out two, J. Loeb and H. Fitting, accumulated facts which demonstrated the action of correlation carriers. But all remained isolated instances which only during the last 20 years could be integrated.

Loeb (14), the zoologist, became fascinated with the regeneration of buds on severed leaves of *Bryophyllum* and always had his laboratory window sills full of those plants. For years he experimented with them, came to the conclusion that this regeneration, the geotropic response of the stems, and the outgrowth of axillary buds were all regulated by hormones, and in several papers he presented evidence that these responses all might be due to a single agent. It was impossible, however, to get direct evidence concerning the nature of this agent, and thus Loeb (15) rejected his earlier idea that a plant hormone was involved, and he assumed that all his observations were an expression of a mass action of some food constituent.

In the work of Hans Fitting (10) we see the opposite development of ideas. In an extensive investigation of the transmission of tropistic stimuli in the *Avena* coleoptile he rejected any suggestion that this might be accomplished by chemical or even physical means. He believed that his experiments showed that light induced a polarization in cells, which could be transmitted from cell to cell, even when by complicated incisions there was no continuous linear connection between the stimulated and reacting cells. This was different from the results of Darwin,

Rothert, and others, and it seemed to disprove the existence of a correlation carrier, or of electrical transmission of a stimulus. This hopelessly confused the issue, so that for years its solution was impossible.

But a few years later when Fitting (11) worked in Buitenzorg, Java, on the flowering of orchids, he found that the swelling of the ovary and the fading of the flowers after pollination was due to a water-soluble, heat-stable substance, which occurred in the pollinia, and which he compared with a hormone. It is unfortunate that this paper of Fitting, which so clearly showed the existence of a hormone-like compound in the plant and proved that it could be handled outside the plant body like any other chemical, had so little influence on botanists, whereas his paper on the transmission of the light stimulus, which we might designate as almost contrary to the facts as we know them now, had such a profound influence.

It was only natural that the work of Fitting stimulated Pfeffer, and he assigned Boysen Jensen, who was just then visiting his laboratory, to repeat Fitting's work. Whereas Fitting had made deep incisions in grass coleoptiles in many different ways without preventing transmission of the phototropic stimulus, Boysen Jensen went one step further. In a few experiments he completely severed the tip of an *Avena* coleoptile and then replaced it. This drastic treatment did not prevent the transmission of the phototropic stimulus either. This experiment invalidated Fitting's hypothesis of transmission of a polarity of the coleoptile cells induced by light.

Four years later Boysen Jensen (3,4) published a full account of his experiments in Pfeffer's laboratory and of additional work in Copenhagen and discussed their implications. Within the framework of current ideas, especially of Pfeffer and his school, the experiments did not fit very well. According to them transmission of a stimulus was a complicated process, comparable to the transmission of stimuli in animals, with the tacit implication that some electrical phenomenon was involved. This seemed to be contradicted by Boysen Jensen's experiment, but in its discussion it was pointed out that potential differences could arise by concentration differences.

Thus in explaining transmission of the phototropic stimulus Boysen Jensen arrived at the mental picture of the transmission of a concentration gradient of substances, which could pass a cut and which could produce the necessary electrical gradient in the base of the coleoptile.

This means that Boysen Jensen definitely thought in terms of diffusible materials, either substances or ions, but that the complexity of the prevailing views on stimuli in plants prevented him from assuming any connection between such substances or ions and simple growth. Not only Boysen Jensen failed to see this connection, but so did all his contemporaries, including Pfeffer. It seems that on the whole more significance was attributed to Fitting's experiments, which were published in much greater detail, and the decapitation experiment was considered a curiosity. Many investigators tried to explain it away, pointing out that since decapitation involved very serious wounding, the transmission might, therefore, be simulated by wound reactions.

Pfeffer was also much worried by Boysen Jensen's experiment, and he induced another visitor to his laboratory, A. Paal from Budapest, to repeat it. This Paal (16,17) did in great detail, varying the experiment in every conceivable way. And even a modern statistician would have been satisfied with the total numbers of plants used. The most important thing, however, was that Paal probed deeply into the nature of the transmission and found that it was just a case of unequal distribution of a growth-promoting substance, which is formed in stem tips all the time. This liberated plant physiology from some of the mysticism connected with tropisms and the stimulus concept. It opened the way to many new and interesting experiments, and it was the first generally accepted demonstration of the existence of a correlation carrier in plants.

We come now to the second stage in the development of our knowledge about plant hormones. This really started with the work of Paal, who clearly stated that in the normal coleoptile tip a growth-promoting substance was formed continuously, which regulated the growth of the cells below the tip. This, and not Boysen Jensen's work, broke the spell cast by Fitting and so many others on the problem of the transmission of the phototropic stimulus. Many investigators, such as Stark, Drechsel, Seubert, Söding, Brauner, and Nielsen, started to work on growth-regulating substances in stems and coleoptiles, all within a few years from the publishing of Paal's paper. Everything pointed towards a speedy solution of this problem.

Since so much of the basic knowledge about tropisms in plants had come from my father's laboratory in Utrecht, and since at the time the growth-regulating substance seemed to be particularly important in the understanding of tropisms, it is obvious that this substance was much

discussed among staff and students. It was partially to settle an argument that I did my first experiment with auxin. Paál had pointed out that there were three possible explanations for a positive phototropic curvature in grass seedlings: 1) decreased production of the growth regulator in the front side of the coleoptile tip; 2) decreased translocation along the front side; or 3) destruction by light along the front side of the coleoptile. Some of my fellow students were in favor of this third possibility, which seemed to be incompatible with many facts as I saw them. If the growth regulator could be handled outside the plant its light stability could be investigated. Therefore, I devoted most of the free evenings and nights which my military training left me to experiment with *Avena* seedlings in the laboratory darkroom. Those were exciting nights when the effects of decapitation and regrafting of the tip were studied and it was gradually revealed that the wounding as such did not have such a severe effect as had been supposed. And then on the night of April 16, 1926, the first coleoptiles made their bow to the tip diffusate which had been collected in gelatin. By 3:00 A.M. the negative auxin curvatures were clearly visible, and it was hard for me to realize that this momentous experiment could not move my father to get up in the middle of the night and accompany me to the laboratory! This work was reported for the first time by my father at the International Botanical Congress in Ithaca (26). I then worked out a quantitative method for the assay of this growth-promoting substance or auxin (28), as it was later named by Kögl and Haagen-Smit. In this way a good basis was laid for a further physiological and chemical analysis of the auxin and its effects.

In a short time some important principles were established: without auxin there is no growth; and as a corollary of this, the Cholodny-Went theory of tropisms, saying that tropistic curvatures are due to differential distribution of auxin within the responding stem or organ.

It is typical of the timeliness of the subject, that first in 1926 for geotropism, and then in 1927 for phototropism, Cholodny (5,6) published two papers assuming, on theoretical grounds, that gravity or unilateral light deflects the normal symmetrical downward stream of auxin. And each time a paper of mine (27,28) was in press giving experimental proof of this assumption.

Concerning the role of auxin in the plant, Dolk found that after decapitation of a coleoptile the reduction in growth rate was due to

removal of the auxin-production center, and that auxin was produced again at the cut surface two and one half hours after decapitation, simultaneously with a rise in growth rate of the stump. Avery *et al.* (1), and W. Zimmermann (31) found a close parallelism between auxin production by the terminal bud of woody branches and their growth rate. Went and Thimann (29) found the same parallelism in the *Avena* coleoptile. It should be stressed that all this work was carried out by measuring the auxin which diffused from the tissues into agar.

It turned out that the responses to gravity of all plants which were investigated fitted excellently into the general auxin theory of tropisms. The work of Dolk (9) should be mentioned here specifically.

Thus the morphological polarity existing in the plant turned out to be based on the polar transport of auxin, and a typical morphological problem was brought into the realm of physiology. It seems amazing that morphologists and physiologists have not made more use of this common meeting ground.

It would take too long to follow in detail all the work which was carried out in the thirties. First a period of consolidation set in, when the knowledge gained about auxin was extended in breadth. In numerous talks and speeches my father disseminated the new knowledge, especially in Europe, so that soon botanists there were auxin-conscious. In America it took longer, and it was really my predecessor in Pasadena, Herman Dolk, who brought the experimental attack on auxin to this country. With Thimann he did pioneer work, and their first two students, James Bonner and Folke Skoog, of course followed an auxin career. Yet in those early days the existence of auxin was still questioned by many otherwise well-informed scientists. Then it was almost an adventure to give a talk on auxin before a botanical audience; the reactions ran the whole gamut from enthusiastic acceptance to disdainful rejection. It is remarkable to observe here, how this situation has changed in the last fifteen years.

Gradually a differentiation in the research on auxin set in. Originally the main emphasis had been laid on a study of its biological and physiological role, its function as correlation carrier, as chemical messenger. This research had opened remarkable vistas of the whole regulation of plant growth; it had shown that a single agent, auxin, tied together a large number of activities inside the plant. It had become clear that the effects of stem tip and young developing leaves on stem elongation were

wholly exerted by auxin; that this same auxin was the agent which caused
apical dominance in stems, preventing the lower lateral buds from grow-
ing out. Auxins were intimately tied up with root initiation, fruit devel-
opment, leaf and fruit abscission, and many other phenomena. Morpho-
logical polarity in organ formation was apparently largely due to polar
auxin transport. On the cellular level auxin influenced a number of
protoplasmic properties and activities, such as permeability, viscosity,
water uptake, cyclosis, etc. Although none of these phenomena were
completely and exhaustively studied, the evidence was so strong that the
participation of auxin hardly seemed a problem any more. Yet if we
consider in detail the physiological role of auxin in particular cases, we
find that there are more discrepancies than we suspect when we view
the problem as a whole. As a particular case I might mention the role of
auxin in phototropism. After the first main objections against the
Cholodny-Went theory were allayed through the work of van Overbeek,
who bridged the chasm between this theory and Blaauw's by showing
that both principles did coexist in a single plant, the problem seemed
almost solved, especially when the carotene activation of auxin-a destruc-
tion by light was found. But now in view of the stability of indoleacetic
acid towards small amounts of light and many other conflicting facts
it seems necessary to reconsider the problem of phototropism from an
auxin angle.

The work on the chemical nature of auxin in the plant, so brilliantly
initiated by Kögl and Haagen-Smit (12) in Holland, and Thimann (23)
in this country, led to developments along two different lines. In the
first place the discovery of the activity of indoleacetic acid and many
related substances as auxins, by Kögl and Haagen-Smit (13), and its
extension in this country by Zimmerman and Hitchcock (30) and many
others, notably Norman and collaborators (25), made it possible to
apply growth-promoting substances, and later, related growth-inhibiting
and herbicidal substances, in concentrations far beyond what the plant
tissues are normally subjected to. This led to what has often been
referred to as plant pharmacology and made practical applications
possible.

The other development which grew out of the chemical work is the
biochemical study of indoleacetic acid inside the plant. Since Haagen-
Smit and collaborators, and Avery and Berger, isolated and chemically
identified indoleacetic acid in extracts from plants it has become bio-

chemically very attractive to study its production, source, and fate. This is now being carried out by many groups of investigators, notably Bonner and Wildman, and Thimann and co-workers. It is now evident that tryptophan can act as a precursor for indoleacetic acid in the plant and that enzyme systems exist which carry out this transformation. Thus we can see how in particular instances (for example, the corn endosperm) large amounts of indoleacetic acid can be produced. Another enzyme system widely occurring in plants inactivates indoleacetic acid. Thus we have the possibility of a complicated interplay between these enzyme systems which gives us a fine opportunity to explain physiological phenomena. This has not been done as yet, so we do not know to what extent the new biochemical intelligence is able to explain the physiological phenomena of growth and correlation.

There is a basic objection against these biochemical studies of indoleacetic acid inside the plant. It is usually tacitly assumed that indoleacetic acid is the one auxin in plants, ignoring all the evidence for the role of substances like auxins-a and -b. Thus a necessarily one-sided and incomplete picture is obtained of the biochemistry of auxin.

Another development has had a decisive influence on the auxin field. That is the use of organic solvents for extraction of auxin. In the earlier work auxin was obtained only by diffusion from the producing or transporting tissues. This had the advantage that only the auxin on its way as correlation carrier was caught and measured. But it was impossible to make a balance of the source and fate of auxin. Thimann (22) succeeded in extracting auxin from *Avena* coleoptiles with chloroform, which later was replaced with peroxide-free ether. First this method gave most interesting results, showing that as a coleoptile grew the extractable auxin disappeared proportionately with the amount of growth. It also could be shown that less auxin could be extracted at any one time than could be obtained by exhaustive diffusion, indicating continuous production and utilization of auxin. It could also be shown that the diffusible and extractable auxin were correlated with completely different phenomena inside the coleoptile. The diffusible auxin bore a direct relationship to tropisms, whereas the correlation between extractable auxin and growth was as direct as Thimann and Bonner (24) had found in their earlier studies.

The extraction method, however, soon degenerated into a race for the most auxin that could be extracted from any one object. The

exhaustive extraction became a fad and was in no way correlated any more with the physiological role of auxin. From all we know about indoleacetic acid and its formation from, for instance, tryptophan, we can easily see that under certain circumstances large amounts of indole-acetic acid could be released from tryptophan by way of protein break-down, which during the life of the tissue never would have been available. I would like to urge not the abandoning of different extraction methods, but their simultaneous use coupled with a physiological analysis. Thus it may be possible to find for each process a form of auxin or a fraction with which it is correlated. In this way it may be possible to make some sense of the enormous amount of experimental data which are amassing.

Let me make clear what I wanted to say with a simile. Suppose that we want to find the role of water in a steamship. By judicious experiments we can find that a certain amount of fresh water is necessary for steam generation, and sea water is needed for cooling purposes. Such experiments would be physiological (for example, plugging the supply line for cooling water, excision and regrafting of fresh water tanks and so on). A biochemical study of the steamship would presumably start with sectioning of the ship into segments and squeezing each. Some sections would yield large amounts of water (those containing the fresh water tanks and ballast tanks), others would have intermediate amounts, such as the boiler room, and again others would yield almost no water, such as the turbine room. Only by judicious separation of water from boilers, steam from turbines, and storage tank water could such a biochemical analysis of the steamship yield intelligible results. Total extraction of water would not give any correlations with the functions of the steam-ship.

The development of the auxin field is a typical example of how science works. Originally growth of a plant was considered almost as a category, in the way the Greeks considered water, fire, or earth. It was taken as a property of the living organism. When it turned out that in the absence of auxin there was no growth, and that stem growth could be controlled at will by the application of measured amounts of auxin, it seemed to many that we had an explanation of growth. This was partly because some of the mystic quality of growth was possessed by a relatively simple substance which could be stored in crystalline form on the chemical shelf of the laboratory. Some of my more mystically inclined friends actually disapproved of the idea of an unromantic chemical

being involved in the growth process. To convince them of the physical reality of the growth-promoting substance, I had to do the diffusion rate experiments proving that the coleoptile tip extract was not an imponderable emanation, but something possessing the common attributes of chemical substances.

After the first enthusiasm for the growth hormone had worn off, it became generally recognized that auxin was not a panacea or the stone of wisdom, but simply that we had pushed back the frontiers of science slightly, creating a much longer and more complex line demarcating fields of knowledge and the unknown. But at the same time it should not be forgotten that even though the discovery of auxin complicated knowledge about growth, it also produced links between many different botanical fields of endeavor, and it brought chemists, horticulturists, anatomists, physiologists, and many others together into a strong unity of common interests.

It is sometimes said that botanists are not sufficiently aware of the practical implications of their work, or are staying too much aloof from practical applications, or choose impractical problems to work on. All these criticisms are either not based upon fact or are not germane. I can assure you that at present only a small percentage of all the practical auxin applications we were talking about twenty-two years ago have materialized as yet. But a botanical laboratory is usually not rich enough nor equipped with large enough experimental fields or greenhouses to carry out the semipractical experiments necessary to apply theoretical knowledge.

The criticism of the impractical subject matter of botanical research can easily be disposed of by pointing towards the auxin research. In the early years all auxin work sprang from previous work on phototropism in seedlings. This was considered so unimportant by more practically inclined botanists, that the plant physiology textbook used most extensively in this country fifteen years ago did not even mention the word tropism. And not only in this country, but also in Holland and elsewhere, auxinologists were criticized for their use of anemic etiolated seedlings. We should be aware of the fact, however, that not until the advent of the completely air-conditioned greenhouse or artificially lighted culture room, were really conclusive experiments with fully mature plants possible. The basic principles of growth are not changed by the growing conditions, but quantitative research requires reproducible plant ma-

terial, and that was available inexpensively, conveniently, with minimal space requirements as seedlings. Such seedlings will continue to be an ideal source of experimental material, and only for processes typical of the mature plant, such as flowering and fruiting, will we be restricted in our experimental material to mature plants.

History is not something which abruptly ends. If I had written my speech an hour ago, I would have had to include the advances made in the auxin field this morning and afternoon. I think all of us this morning were aware that history was being made. Something which had been coming slowly, almost imperceptibly, was suddenly spotlighted. I refer here to the demotion of auxin by Dr. Thimann from an executive position to a simple policing job. This actually creates a number of new positions to be filled, and I hope that soon not only nominations for the position of Director of Root Formation, or Coordinator of Cambial Activity will be made, but that in competitive experimental examinations these positions will be filled by purified and recrystallized chemicals, which will take a place of honor on the shelf of any self-respecting chemist, and which will become known by some cryptic combination of letters and numbers.

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Plant Hormones in Practice

P. W. ZIMMERMAN

As a concept lying along the frontiers of science, plant hormones appeared on the horizon with the publication in 1880 by Charles Darwin of a book called *The Power of Movement in Plants*. Startling advances have been made and in a way each new phase of the subject has constituted a new horizon.

My assignment today concerns a chemical revolution in science and horticultural practice; that is chemical regulation of the pattern of growth and development of plants. The subject is new but the progress has been rapid. The chemicals involved are called hormones, auxins, or growth-regulating substances. The early experiments and results were looked upon as laboratory curiosities, but they finally led to botanical, agricultural, and horticultural applications which are sweeping the world today. Owing to the varied types of responses induced by plant hormones, the subject drew the attention of botanists, horticulturists, and chemists. Professors in universities found the field a fertile one for graduate students and assigned projects which resulted in published theses for advanced degrees. Publications are now pouring out at a rapid rate.

No one person or small group of persons could ever have discovered all the facts that are now known about plant hormones. It is hardly conceivable that extensive horticultural applications could have arisen from the simple but profound researches involving the bending of the oat coleoptile exposed to unilateral illumination and the realization that this was due to an influence of a chemical nature. It was by no means easy for scientists to agree on the meaning of such growth responses, but through fear, envy, suspicion, interest, and curiosity one worker picks up where another leaves off and advances our sphere of knowledge.

However, no man's word is law; all claims must be tested and proven before they are acceptable in science.

Interest in the subject of plant hormones from the standpoint of fundamental science is still uppermost in the minds of many workers. It is increasingly evident, however, that an attack on practical problems in the field of plant hormones also contributes to fundamental science. It may be said that when practical problems are properly studied, the results of the investigation contribute to both science and practice. Laboratory curiosities pointed the way to horticultural applications. To show the wide interest in the field of plant hormones, the following titles of publications which recently came to my desk are taken at random:

> Inducing fruit set and seedless tomatoes
> Hormones retard bud development
> Hormone sprays and their effect upon the shipping and keeping quality of Bartlett pears
> Effects of certain growth-regulating compounds on Irish potatoes
> Inhibition of bacterial growth by auxins
> Apple-bloom thinning with chemicals
> 2,4-D hits cotton again
> New weed killer plant for Australia
> Direct introduction of chemical substances into herbaceous plants
> 2,4-D injury to trees
> Spraying is a new method of applying root-promoting substances
> The use of 2,4-D in rice fields for the control of weeds
> Quack grass conquered by new chemical powder
> Method of defoliating
> The enzymatic inactivation of indoleacetic acid
> Synthetic plant hormones and the pineapple industry
> Foresee new uses for 2,4-D

The groups of chemicals best known as growth regulators are as follows: Indole compounds involving β-indoleacetic and β-indolebutyric acids; naphthalene compounds involving α-naphthaleneacetic acid and its derivatives, β-naphthoxyacetic and β-naphthoxypropionic acids and

their derivatives; substituted phenoxyaliphatic acids especially 2,4-di-chlorophenoxyacetic acid (2,4-D) and higher homologs, α-(2-chloro-phenoxy)propionic acid, 2-methyl-4-chlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid and higher homologs; and unsaturated hy-drocarbons, especially ethylene and acetylene. While there is some varia-tion in uses and responses induced with acids, salts, esters, and amides, for our purpose they may be thought of as similar in bringing about desired results. There are many natural hormones which have not been isolated and definitely identified but nevertheless are known to exist. Indoleacetic acid and ethylene are two compounds manufactured by plants and definitely identified by chemical methods.

Today there are many lines of research as indicated by the titles of publications just read. From the fundamental research angle workers are trying to find how hormones work and how they are synthesized. Much emphasis is being placed on enzymatic activities, precursors, and reactions ultimately leading to active substances. Another group is trying to harness plant hormones and put them to work. Physiological activity is found with simple structures like ethylene and with the more complex structures like naphthaleneacetic acid and substituted phenoxyaliphatic acids. New compounds are being discovered almost daily, and it is likely that the most effective ones are yet to come.

Methods are adapted to meet the needs for each particular kind of work to be accomplished. New and better methods are constantly being developed. Among others the following applications resulting from plant hormone research have been established:

Propagation of plants through hormone treatment of cuttings and scions.

Prevention of pre-harvest drop of fruit, especially apples and oranges, with naphthaleneacetic acid or 2,4-D.

Increasing fruit set and induction of seedless tomatoes with numerous hormone-like substances.

Inhibition of buds to prevent potatoes from sprouting by the use of vapors from esters of naphthaleneacetic acid.

Inhibition of fruit tree buds or prolonging dormancy to pre-vent loss through late frost though the methods are as yet not well perfected.

Inhibition of buds of nursery stock to prevent growth until the young plants are established.

Regulation of flowering of pineapples by the use of ethylene, acetylene, naphthaleneacetic acid, naphthoxyacetic acid, and substituted phenoxy compounds. In this way a crop can be staggered to facilitate harvest and canning operations and also increase size of fruit of certain varieties.

Defoliation of plants with ethylene, acetylene, propylene gases, and other chemical means.

Prevention of leaf fall in contrast with defoliation.

Thinning of fruit, especially apples, with hormone sprays bringing about better size and quality of fruit and causing biennially bearing trees to bear annually.

The use of hormone-like chemicals as selective weed killers.

Defoliation and inhibition of growth were given great prominence during the war. It is conceivable that chemicals may be developed which can be sprayed on the forest to defoliate the plants and thereby expose the enemy. Crops may be killed over large areas by the use of chemicals dusted or sprayed by means of an airplane or allowed to float over the fields through artificial fogs or smudges.

Extensive use of plant-hormone-like substances became evident during the past year when the sale and use for 2,4-D practically equaled that of DDT which has been leading the list of all other organic agricultural chemicals. α-Naphthaleneacetic acid was also high on the list since large orchards were sprayed to prevent preharvest fruit drop.

One of the best illustrations of growth regulation of plants is the modification of the pattern of new organs growing under the influence of applied hormone-like chemicals. Substituted phenoxy, benzoic, and naphthoxy acids are particularly effective for this purpose. Mature organs do not change their shape, but all new organs which grow under the new chemical influence are modified. This leads to the assumption that normal leaf patterns are determined by natural chemical influences within the plant. Under new and stronger chemical influences the leaves and other organs assume a new pattern. So far no practical applications for this odd response have been found. However, we should not be surprised if one crops up since many other curiosities in the plant-hormone field have eventually led to practical applications.

To date no hormone-like chemicals or any chemical alone have been found to stimulate growth of the entire plant in the same sense as is

recognized for complete fertilizers. The scant claims for such stimulation have not stood the test of time.

Flowers of solanaceous plants last for an abnormally long time after being treated with hormone-like substances which induce parthenocarpic development. This fact is very suggestive and gives the idea that the life of cut flowers can be prolonged. To date, unfortunately, these phenomena seem to apply only to intact plants. No effective means for substantially increasing the life of cut flowers have been discovered. Attempts, however, to find methods or chemicals for this purpose should not be discouraged.

From our experience with substances which induce roots, modify leaves, and otherwise regulate growth, it appears evident that all organs of the plant are under some regulating influences probably of a chemical nature. It would seem, therefore, that we should find flower-inducing substances and shoot-inducing substances. While there have been various claims for shoot-inducing substances, this is not a reality in the same sense that we have root-inducing substances. There is a special case reported where leaves of the pineapple plant produce many adventitious buds after treatment with 2,4-D. Other species have not responded in like manner. It is logical, however, to assume that such chemicals do exist in nature and eventually may be found. Once located, if they ever should be, progress should be rapid and results spectacular.

One of the greatest single problems in agriculture and horticulture is the control of weeds. For many years one of the farmer's greatest problems has been cultivation largely to rid the field of weeds. However, that situation is changing rapidly since the discovery that hormones can be used to regulate and actually kill troublesome weeds. Lawns can be sprayed to rid them of such weeds as dandelion, plantain, and hawkweed without killing the grass. Interestingly enough, 2,4-D can be applied along with fertilizer to kill the weeds while the grass is being stimulated.

Weed killing is by far the most important practical use of hormone-like chemicals. The second in importance is the use of naphthaleneacetic acid to prevent preharvest drop of apples. 2,4-D alone has a potential annual market of more than 100,000,000 pounds and may soon top the list of all organic agricultural chemicals.

There are also other achievements with plant hormones, and these illustrate the varied lines of attack from the practical point of view. In Hawaii several kinds of plant hormones are being used to force

pineapples into flower or to increase fruit size. Still other hormones prevent premature flowering of pineapples, and finally the vegetative propagation of pineapples is facilitated with plant hormones. They are rapidly becoming a part of our everyday life, and, as with the telephone and the X-ray, we shall soon wonder how we ever got along without plant hormones.

Fortunately monocotyledonous species are more resistant than many of the dicotyledonous species. Since corn covers such a vast area of land, it is encouraging to find that at least some types of weeds can be controlled in the field without harming the corn. The results reported are somewhat variable but sufficiently promising to warrant further testing. It may develop that certain varieties are resistant enough to permit sufficient amounts of 2,4-D per acre to kill noxious weeds without affecting corn. Treatment of the soil after planting and before weed seeds have started may be the answer to some of the problems. This pre-emergence treatment applied after the corn is planted appears to control both broad-leaved annuals and weedy grasses. One to five pounds per acre can be applied as a spray on the ground without preventing corn from germinating and growing. If applied after corn starts growing, care must be taken to hit at the base of the stalk rather than at the tops. When tops are sprayed the corn leaves fail to separate and remain rolled up so that the tassel must push out at the side. Also abnormal flowering and prop roots appear, and the crop may be decreased. However, the results are sufficiently promising to indicate that with selection of proper varieties of corn and improvement of methods controlling weeds in a corn field will be a reality.

Weeds in sugar cane fields, particularly alligator weed, have been controlled by applying 0.2 per cent of 2,4-D at the rate of 100 gallons per acre. Both dusting and spraying were done without noticeable decrease in the sugar production. Weed control in the tropics was demonstrated by van Overbeek and shown to be of tremendous economic importance.

There are some 10,000 miles of waterways in the southern district in and around the state of Louisiana which have become infested with water hyacinth and alligator weed. This constitutes a major problem assigned to the army engineers of the southern district. Navigation, drainage, health of people and wild life are all affected by this problem. There are a number of contact weed killers which are effective on aquatic plants, but they are poisonous to fish and other animals and also

hazardous to humans. Consequently the use of hormone-like herbicides appears to be the most feasible method for controlling water weeds on a large-scale basis. Working with the army engineers during the last two years, Hitchcock, Kirkpatrick, Earle, and I have shown that waterways can be kept free of aquatic weeds by means of 2,4-D sprays and proper maintenance control. The pounds per acre, methods of applying the spray, and weather conditions are all important when the first application is made. Complete eradication may not be possible, but practical patrol maintenance is feasible and keeps weeds within bounds. By means of a helicopter the equivalent of 600 acres per day can be sprayed at a cost of fifty cents an acre for the use of the plane. The plane was used on the marsh lands as well as waterways. The results are striking, and large-scale applications of hormone-like herbicides on forest areas, cereal crops, and swamp land can be predicted with certainty.

Let us try to look beyond the present horizons for plant hormones and predict some of the things to come. We shall have hormone-like selective weed killers for every conceivable use—for corn fields, wheat fields, orchards, gardens, forests, waterways, swamp lands, hedge rows, and yards. Any hormone which has specificity in its effects is likely to find special uses. Perhaps the forest can be sprayed to kill all but pines or other desired species. With a little stretching of the imagination we can picture a lawn without crab grass, a bayou in Louisiana without water hyacinth and alligator weeds, pastures without thistles, onion fields not weeded by hand, and helicopters available for spraying at twenty-five cents an acre.

Bud-inducing chemicals are seriously needed. They would facilitate propagation of plant parts where buds have not appeared or where they have been lost. Day lilies, Gloriosa lilies, and dahlias are often lost because the storage organ does not have a natural bud. Internodes which do not normally produce adventitious buds could be used for propagation purposes. A shoot-inducing substance should cause new shoots to arise where desired on the plant to make possible propagation of budless parts or improve the shape and appearance of intact plants. There is no end to the conceivable uses of bud- or shoot-inducing substances.

Flower-inducing substances which are thought to exist in nature may be isolated, identified, and used as a common tool. If this becomes a reality it should be possible to force long-day types to flower during short days or short-day types to flower during the long days. In short,

it should be possible to induce flowering of plants at will and to force flowers to grow at unusual places. We can imagine plants with flowers on internodes, on leaves, and even on roots.

At the present time there appears to be considerable variation in the time of ripening of fruit. Under the influence of chemicals the time of ripening should fit into our needs. During the past season it has been shown that apples treated with certain hormone-like chemicals ripened prematurely. To a lesser degree this has been noticed for tomatoes. Tomato flavors are not affected under the influence of the chemical. Apples, however, change flavor and consistency, but the fact that modifications in time of ripening have been demonstrated offers encouragement for practical methods applicable to all or many fruits.

Since it has been demonstrated that fruit buds can be delayed through treatment with growth substances, one is led to the assumption that fruiting of tropical species and flowering of plants in general can be staggered to extend throughout the entire season. Mangoes, for example, flower and ripen fruit at definite periods of the year. During the rest of the year they are not available as food. Since this is an important tropical food, it would be desirable to extend it throughout the season. This should become a reality by the proper hormone applications to growing buds. The idea is particularly applicable to tropical plants because the temperature and other growing conditions would not limit the time of fruiting. We should, however, be able also to stagger flowering of spring shrubs in the north so that we may enjoy them over longer periods.

Without making further predictions, it appears that the field of plant hormones presents a challenge for scientists with varied attacks. From the fundamental research angle, it might be said that the work is just beginning. We have found only a few natural hormones, and we still know little about how hormones work. From the standpoint of horticultural applications progress has been more rapid than in fundamental research, and the results so spectacular that interest in the subject is now sweeping the world. Research holds much for the future and more important applications are sure to be made.

There is much competition in the fields covered by this paper. Perhaps that is why progress has been rapid. Research in some phases of the subject can be done by amateurs and practical horticulturists as well as by trained scientists. It is well to recognize the fact that several techniques have played a part in solving difficult problems. We take

new courage when we remember that this is the age of research. We must follow where research leads. Thousands of researchers are looking into the unknown or fields that have no other limits than man's imagination. Research is not confined to the laboratory. Its origin is in the individual. All of us are researchers no matter what our jobs, and we must surpass what has already been accomplished to keep ahead. We must search for new and better methods, for even that which we now do well must be done better tomorrow.

Growth Substances
in Plant Metabolism

The Study of Growth Substances in Plant Metabolism

R. H. BURRIS

To a group such as this the title listed on the program, statement of the problem, must appear superfluous. I think this is particularly true in view of the fact that Dr. Thimann's paper constitutes a superb statement of the problem in addition to an answer to certain phases of it. Perhaps it would be more accurate to say that we would now present a restatement of the problem.

Our concern today is to describe in biochemical terms the mode of action of plant growth substances. It is immediately apparent that this is a big order and one which, in our present state of knowledge, we can complete in a sketchy manner at best. However, an integration of current information and a projection of this into working hypotheses may be useful in directing subsequent efforts.

In dealing with plant growth substances we must first conscientiously define the particular effect in which we are interested and then employ caution when we translate results obtained there to explain effects of a different nature. This shifting is analogous to shifting from lane to lane in traffic, unless you signal your intentions to others you are likely to be bumped ignominiously in the rear.

The need for caution in definition of effects and transfer of information from one effect to another arises from the multiplicity of actions of plant growth substances. Not only will a given substance cause different actions on various tissues but even more intriguing is the fact that a variation in the concentration of a growth substance not only will cause a quanti-tative variation in the intensity of a plant response but also may cause a qualitative change in the response. This property is not confined to plant growth substances, for many inhibitors, for example cyanide, may exert a stimulation at very low concentrations. However, the variety

and range of responses elicited by plant growth substances appear to be unique.

With due caution to the definition of effects studied and experimental conditions employed, we now return to the question of how can we describe in biochemical terms the mode of action of growth substances in eliciting particular responses in plants. As Dr. Thimann has pointed out, the minute quantities in which growth substances bring about their most interesting effects immediately suggests that they are not acting as substrates but in all likelihood are serving as cofactors, constituents, or antagonists of enzyme systems. If we accept this point of view we can rephrase our basic query as, how can we describe in biochemical terms the effect of growth substances on the enzymatically catalyzed processes of the cell. This rephrasing does not simplify our problem particularly, for it does not define which of the multiplicity of cellular enzymes we must examine for growth substance effects.

Our reasoning to this point forms a rather logical approach to our problem. But now we are confronted with the necessity of choosing one particular enzyme or group of enzymes and determining whether it can be related to any particular effect of growth substances. Let us, for example, choose herbicidal action as the growth substance effect. What enzymes shall we study? Data in the literature suggest that herbicides cause the mobilization of carbohydrate in the plant, so we might logically test the influence of various concentrations of herbicides *in vitro* on amylases. Other reports show an increased percentage of nitrogen in seeds from plants treated with sublethal doses of herbicides. The herbicides may influence nitrogen anabolism or catabolism, or the observed effect could merely reflect an enhanced carbohydrate depletion. Enzymes concerned with transamination or reductive amination might logically be examined. A considerable number of reports indicate that herbicidal action is accompanied by increased respiratory activity and carbohydrate depletion. In contrast we find that herbicides in high concentrations may inhibit respiratory enzymes. Hence, we can adopt either of two working hypotheses; first, that herbicides kill a plant by stimulating its carbohydrate oxidation to a point where it burns itself out, and second, that a herbicide acts by directly inhibiting respiratory processes.

These two opposite possibilities reveal another problem which besets the investigator—namely, the type of test material to employ. In general, although there are exceptions to this statement, the concept of herbicidal

action by enhanced respiration has been derived from observations of intact plants, whereas the idea of herbicidal action by respiratory inhibition has arisen from studies of tissue slices or cell-free enzyme systems. We must accept Dr. Went's warning that the percentage of water in any given segment of a steamship does not necessarily define the role of water therein. Whenever possible we must devise experimental checks with intact plants to test our observations made with isolated enzyme systems.

Of the enzyme systems investigated for their relation to herbicidal action the respiratory enzymes have received most attention. This is not surprising, for they are perhaps the most intriguing and widely investigated group of enzymes in other regards, and we all admit their vital importance in supplying the driving force for cellular processes. In addition, work with growth substances has often turned up effects which strongly implicate respiratory processes.

In this statement of the problem we have chosen to discuss the mode of action of herbicides, but this has merely served as an example. It is open to question whether herbicidal action and other effects of growth substances stem from an influence on a common enzyme system. We are still some distance from a complete explanation in biochemical terms of the mode of action of plant growth substance. It is our hope that the accumulation of additional data and the synthesis of information in common meetings, such as we have here today, may lead to an elucidation of the basic mechanisms of the many faceted activities of plant growth substances.

Changes in Metabolism During Growth and Its Inhibition

K. V. THIMANN, W. D. BONNER, JR., AND
G. S. CHRISTIANSEN

IN THE growth of isolated sections of *Avena* coleoptiles it was observed some years ago (5) that iodoacetate acts as a powerful inhibitor. A more careful study of this inhibition and the circumstances surrounding it has revealed some interesting facts. The procedure is simple; seedlings are grown on moist filter paper and the coleoptiles decapitated; three sections each 3 mm. long are mounted on combs floating in indoleacetic acid solution containing 1 per cent sucrose. After growth in darkness at 25°C. they are measured, usually at 7, 24, and 48 hours. All conditions are rigidly controlled, for there are numerous possible sources of variability. One of these is the age of the plants from which the sections are cut (10). The younger the plants, the more vigorously the sections grow. However, younger plants are also less sensitive than the older plants to inhibition by iodoacetate. In Figure 1 (from 10) the growth of the uninhibited controls is in each case set at 100 per cent. It will be seen that coleoptiles 54 hours old yield sections which are only incompletely inhibited even at concentrations as high as 10^{-4}M. At the other extreme the sections from coleoptiles 120 hours old show threshold inhibition even at 10^{-5}M, and at 5×10^{-5}M are 100 per cent inhibited. The curves for the intermediate ages show the complication of growth promotion at low concentrations of iodoacetate, but in other respects the sensitivity is intermediate between the two extremes. Thus in general the resistance of the plants to iodoacetate decreases steadily with age.

A characteristic feature of the iodoacetate inhibition is that it is completely prevented by the presence of certain organic acids (5,10).

Malate, fumarate, succinate, isocitrate, and pyruvate are all effective in this way (Fig. 2). Concentrations of about M/1000 are needed. With these acids added to the auxin and sucrose solution the coleoptile sections grow at least as much as though the iodoacetate were not present, and in general somewhat more. Even malonate, which at higher concentrations is itself a growth inhibitor, protects against iodoacetate.
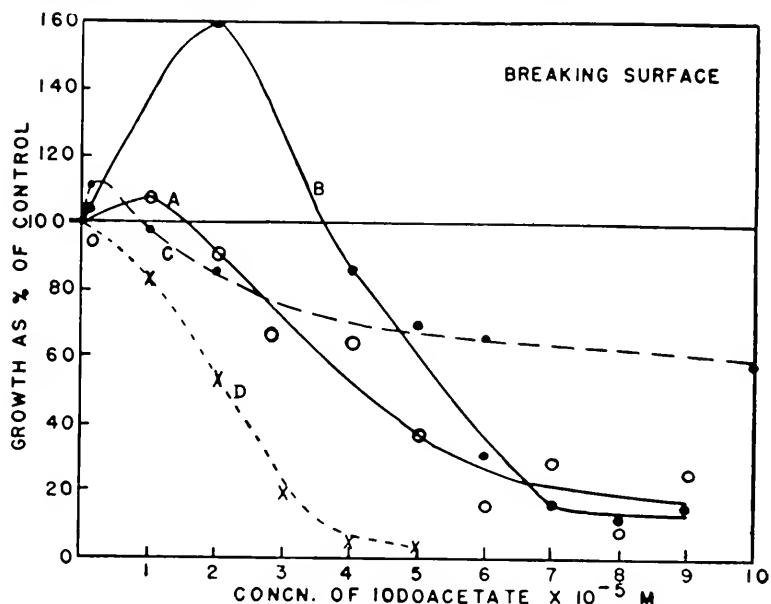
Figure 1. Effect of iodoacetate on the growth of *Avena* coleoptile sections after 48 hours at 25°C. in darkness in indoleacetic acid 1 mg. per liter plus sucrose 1 per cent. Sections cut from plants aged: curve A, 74 hours; B, 64–66 hours; C, 54–56 hours; D, 96 hours. The growth of the uninhibited controls is placed at 100 per cent for each age. (10).

It follows from these observations that the amount of the dicarboxylic acids normally present in the coleoptile must exert considerable influence upon the response of the plant to iodoacetate. If the content of these acids were high enough, presumably iodoacetate would not inhibit growth at all, and if it were to decrease with increasing age, the decrease in resistance to iodoacetate would be explained. Accordingly analyses were made for total organic acids, malic and citric acids, in coleoptiles of different ages. The methods of Pucher and Vickery (8) were used, the same 9 mm. zone of the coleoptiles being analyzed as was used for
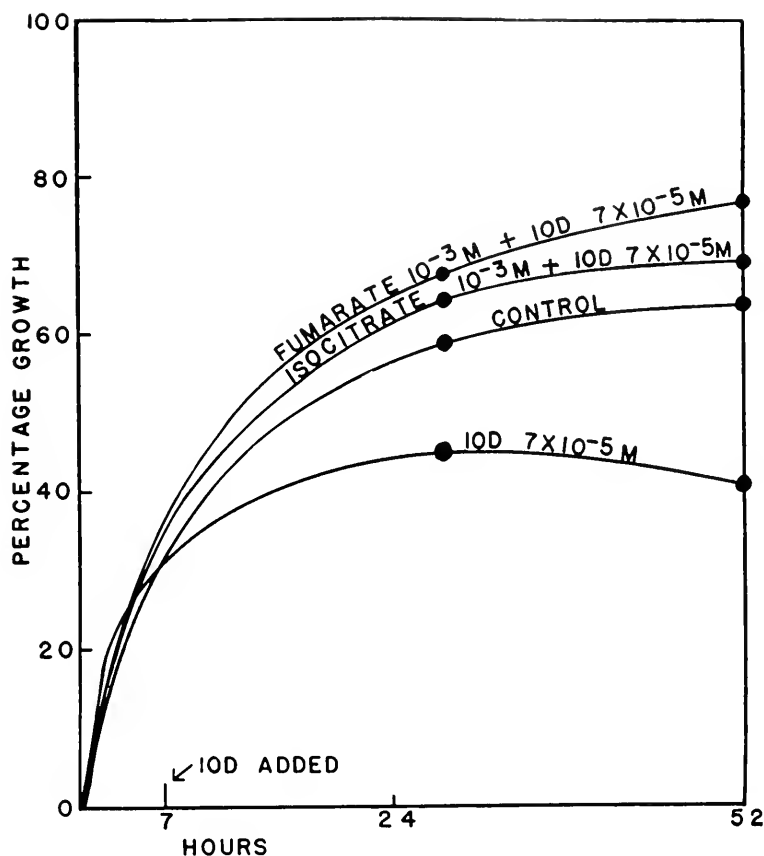
Figure 2. Protection against iodoacetate inhibition. Sections cut from 64 hour coleoptiles, in solutions containing indoleacetic acid 1 mg. per liter plus sucrose 1 per cent. Iodoacetate was added 7 hours after placing the sections in solutions; this accounts for the incompleteness of the inhibition at 7 x 10⁻⁵M. (10).

growth studies. The results (Table 1) show that the above expectation is realized. It is of interest to recall that Sweeney and Thimann (9) some years ago deduced from the study of protoplasmic streaming that the content of organic acids in the coleoptile must decrease with increasing age.

Pea stem sections, at the age used for our growth tests, have a content of malate and citrate equal to that of very young coleoptiles. This corresponds well with their relative insensitivity to iodoacetate, of which a

TABLE 1

Organic acid content of *Avena* coleoptiles as a function of age, and of *Pisum* stems. Plants grown in darkness at 25°. All figures are micro-equivalents per gram dry weight

Age of tissue (hours)		Total Acids (μ-equiv.)	Malic Acid (μ-equiv.)	Citric Acid (μ-equiv.)
Avena Coleoptiles	54	451	205	23
	74	368	169	17
	96	320	85	16
	120	205	60	12
Pisum Stems	7 days	888	192	23

concentration as high as 6×10^{-4} M is needed for 50 per cent growth inhibition.

The question now arises as to whether this disappearance of organic acids is a result simply of time or whether they are actually used up in growth. To answer this question sections were cut and allowed to grow in the usual way in auxin; after measurement they were analyzed for organic acids as before, with an additional test for pyruvic. This work is tedious, involving large numbers of stem sections. A number of experiments were also carried out in which growth was inhibited by iodoacetate or arsenite. Arsenite differs from iodoacetate in that its action is not prevented by organic acids, but it probably reacts with the same sulfhydryl enzyme (11). Some of the results are given in Table 2. Malate, but not citrate or pyruvate, is evidently used up when the sections are

TABLE 2

Organic acid content of pea stems before and after growth. All values expressed as micro-equivalents per gram dry weight

Condition of sections	Per cent growth	Malic (μ-equiv.)	Citric (μ-equiv.)	Pyruvic (μ-equiv.)
Initial	—	202	22	8.8
After 24 hours in:				
Water	19	143	23	7.3
Auxin*	58	115	9	4.7
Auxin + Arsenite	27	190	11	8.2

*Indole–3–acetic acid, 1 mg. per liter.

merely kept in water, that is, when little elongation takes place, but all three acids are used up rapidly when the sections grow. As we shall see below, these acids are indeed not the only metabolites which are consumed in the process of growth. Furthermore it is clear that when growth is inhibited by arsenite, the consumption, especially of malate and pyruvate, is prevented.

Now the metabolism of the organic acids is interrelated with many aspects of the oxidative metabolism of tissues so that the consumption of organic acids in growth, and their preservation when growth is inhibited, would indicate that growth and inhibition involve deep-seated changes in metabolism. Yet it is a curious fact that when growth is promoted by auxin the total oxygen consumption is not increased detectably in the coleoptile,* and only 15 per cent in the pea; correspondingly also, growth can be inhibited without detectable decrease in oxygen consumption. In the case of inhibition by fluoride there is even a small increase in oxygen consumption. These considerations led us to make a more careful study of the changes that go on during inhibition. For this purpose we have used a growth inhibition of 50 per cent as a reference point because, (a) this is achieved with relatively low concentrations of inhibitors, and (b) it takes place with only minor changes in respiration rate. Pea stem sections were used rather than coleoptiles because they do not require added sucrose for growth. The three inhibitors, iodoacetate, arsenite, and fluoride were all used.

We have previously reported (4) that when growth is inhibited reducing sugars disappear. They disappear steadily with time, whether the sections are growing or not, but in the presence of inhibitor (at a concentration sufficient to reduce growth by 50 per cent) their disappearance is accelerated. Arsenite, iodoacetate, and fluoride all had the same effect. Since the oxygen consumption of the sections is not increased, the polysaccharides were determined. However, there was no significant change in any of the cell-wall constituents, and the sections do not contain starch. The sugars, therefore, were neither being oxidized nor polymerized. An examination of the neutral ether-soluble material

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*There is some difference of opinion on this. Van Hulssen (1936), J. Bonner (1936), and Commoner and Thimann (1941) found no increase, while Berger, Smith, and Avery (1946) and J. Bonner (1949) record increases of 20—25 per cent when physiological concentrations of indoleacetic acid are added. Kelly and Avery (1949) found that 2,4-D increased respiration in concentrations below the toxic level.

(fats) was then made, and this revealed the fate of at least part of the reducing sugars. As shown in Figures 3 and 4, the inhibitor causes a large increase in the content of fats in the sections. The extent of the reaction is proportional to the concentration of the inhibitor. The action of iodoacetate is in the same direction but less marked. In the case of arsenite the final fat level is equal to the initial value, but in the case of fluoride there is an actual formation of additional fat. These phenomena

EFFECT OF ARSENITE ON
ETHER   SOLUBLES
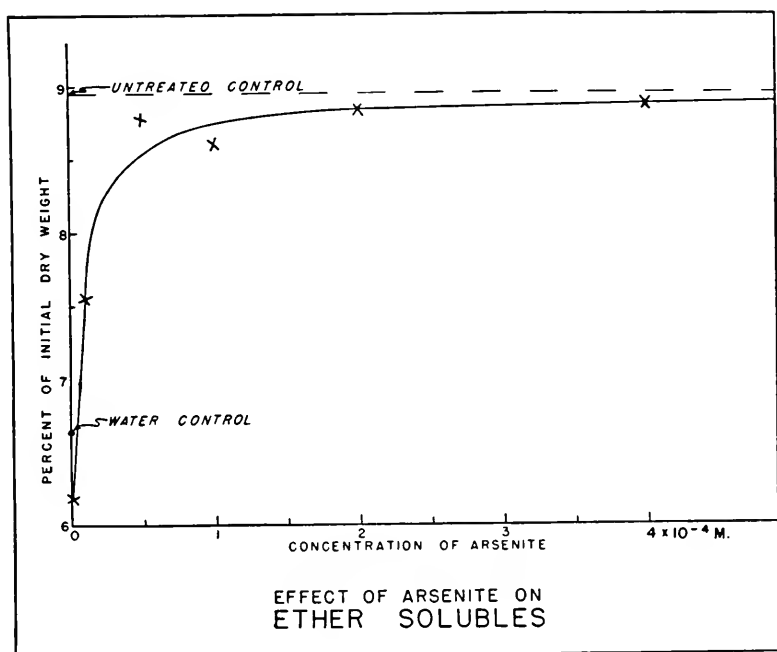
Figure 3. Changes in the fat content (neutral ether soluble fraction) of pea stem sections after 24 hours in indoleacetic acid 1 mg. per liter with and without sodium arsenite. The As concentration giving 50 per cent growth inhibition was 1×10^{-4} M.

indicate far-reaching metabolic changes, the occurrence of which is concealed by the constancy of the oxygen consumption.

Still other far-reaching changes take place in the nitrogen compounds. Analyses for proteins, amino acids, amides, and ammonia, of which a few selected data are given in Table 3, show the following facts: 1) as the sections are maintained in water the amino acids are consumed; 2) this consumption is more complete if the sections are actually growing in

PERCENT OF INITIAL DRY WEIGHT

UNTREATED CONTROL

WATER CONTROL

CONCENTRATION OF FLUORIDE 8×10^{-3} M.

EFFECT OF FLUORIDE ON
ETHER   SOLUBLES
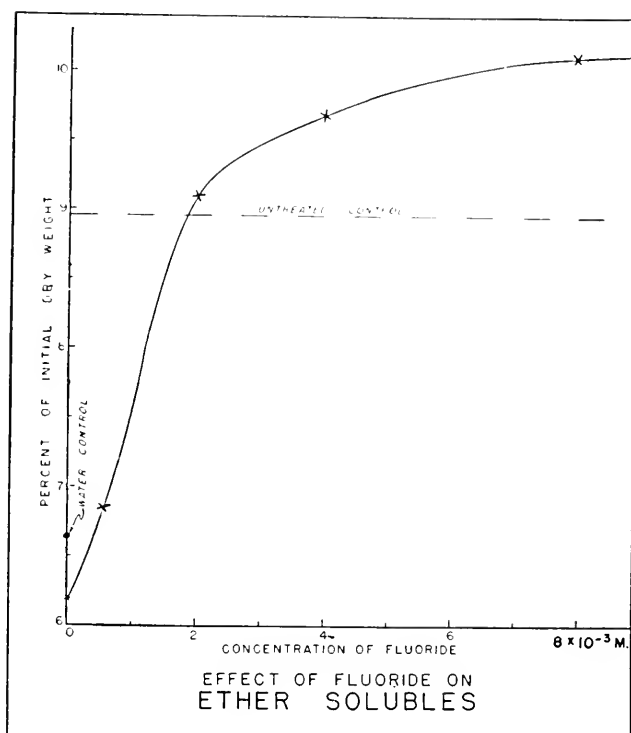
Figure 4. As Figure 3 but with sodium fluoride. The NaF concentration giving 50 per cent growth inhibition was 5×10^{-3}M.

TABLE 3

Changes in nitrogen compounds of pea stem sections during growth and inhibition

All analyses as per cent of initial dry weight

TREATMENT	ELONGATION PER CENT	AMINO ACIDS	PROTEIN (OF PLASMA)	ASPARAGINE
Initial	—	13.7	13.3	6.7
Water control	20.0	1.9	18.9	11.0
Auxin 1 mg./liter	50.9	0.6	20.1	11.5
Auxin plus:—				
Iodoacetate 6 x 10^{-4}M	25.6	6.8	13.4	8.1
Arsenite 1 x 10^{-4}M	26.5	8.7	15.3	7.6
Fluoride 5 x 10^{-3}M	25.3	7.5	16.6	7.7

auxin; 3) the nitrogen of the amino acids is converted partly to asparagine and partly to the protein of the cytoplasm; 4) these changes are in large part prevented by all the three inhibitors.

It is of interest that the process of cell elongation does involve considerable synthesis of protein. It is also of interest that, while there are definite differences, all three inhibitors act, at least qualitatively, in the same way. This might well be expected for iodoacetate and arsenite, since both are sulfhydryl reagents (11), but fluoride almost certainly acts on another enzyme system and hence similarity between its action and that of the others would hardly have been expected.

Lastly it must be pointed out that these results, incomplete as they are in themselves, are consistent with the idea that it is organic acid metabolism which is primarily responsible for growth. For it is only by way of the organic acids that the oxidation of sugars, the formation of fats, and the conversion of amino acids to asparagine are linked. This interpretation therefore agrees well with the concept put forward in an earlier paper, that auxin acts on an enzyme concerned in four-carbon acid metabolism.

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Stimulation of Respiration in Relation to Growth

GEORGE S. AVERY, JR.

THE widespread natural occurrence of plant growth substances is one of the striking physiological attributes of the plant kingdom. From higher plants to lower plants hardly an organism, or any of its parts, has ever been tested and found to be without hormones. This wide distribution of such substances in plants, in the light of their high physiological activity, argues for their having some sort of universal role in metabolism.

There is ample circumstantial evidence to connect these substances with the stimulation of respiration. For example, seeds of certain plants which have been carefully investigated are known to possess a hormone precursor which is hydrolyzed in the course of germination, giving a continuous high level of hormone supply during early growth. This high hormone supply goes hand in hand with the accelerated respiration and growth which is typical of germinating seeds. The growing root and stem tips of plants, as well as the cambium, constitute another example: meristems are regions characterized by the rapid production of new cells, the basis of which is the synthesis of new protoplasm. Wherever new protoplasm is produced hormone concentration is high as are the respiration and growth rates. In spite of these commonly known facts, to date there is surprisingly little experimental evidence linking hormone concentration with the stimulation of respiration, and growth.

Now briefly as to how phytohormones exercise their growth-regulating roles. We have the simple picture that growth responses, in higher plants at least, depend on differential distribution of phytohormones in their tissues and on the relationship of these hormones to other substances which are important in growth. If it is finally shown that hormones exert their growth-controlling influence through respiration, it seems to

become a matter of different rates of metabolism being responsible for different growth responses, such rate differences being due to dissimilar hormone concentrations in various tissues. It has never been demonstrated, however, that hormones occur in tissues in concentrations that would inhibit metabolism. But let us look at the literature on the subject, to see what evidence there may be for linking hormone-accelerated respiration with growth.

The first study of this sort is that of Bonner (6). He suspended small segments of *Avena* coleoptiles in solutions of an impure hormone preparation. At suitable concentrations the segments elongated considerably (over the controls), and this speeded growth was accompanied by an increase in the rate of respiration as measured by oxygen uptake in a Warburg respirometer. The author concluded, "it seems possible that the increase in respiration caused by growth substance may be an essential part of its action in growth." Three years later Bonner (7) again reported on the same general experiments, but this time carried out with improved Warburg vessels and crystalline auxenolonic acid (auxin b) from the Utrecht laboratories. From this work he concluded, in agreement with similar results obtained at Utrecht, that auxenolonic acid failed to stimulate respiration; indeed, a threefold purification of the impure hormone he had used in 1933 removed most of its stimulatory power. So, although growth was stimulated in the presence of the purified hormone preparations, Bonner reported no increase in respiration.

In 1940 duBuy and Olson (11) studied the respiration of single infiltrated *Avena* coleoptiles and found that indoleacetic acid alone or in the presence of fructose had no accelerating effect on respiration. Sweeney (18) pointed out later that the failure to obtain accelerated respiration was caused by the use of water-infiltrated coleoptile segments, which were low in oxygen tension.

In 1941 Commoner and Thimann (10), in an experiment of somewhat similar design, reported a small but significant stimulation of respiration in *Avena* coleoptile segments in the presence of certain four-carbon acids, malic and fumaric in particular. They found that the acids accelerated growth as well as respiration if the hormone indoleacetic acid was present in a concentration of 1 to 10 mg. per liter. When the data were plotted the curves for growth and respiration were found to parallel one another roughly. The authors concluded that "the four-carbon acids provide a respiratory system which is part of the chain of

growth processes, and which is in some way catalyzed by auxin. It represents a small but variable fraction of the total respiration." Here for the first time was promising evidence of hormone stimulation of respiration and stimulation of growth. At this point growth studies seem to have fallen by the wayside for a number of years. Respiration studies, as influenced by hormones, were no longer carried out with parallel fact-finding on growth.

Evidence for the stimulation of respiration with the hormone indole-acetic acid comes from the work of Berger and Avery (1,2,3,4). They found both malic and alcohol dehydrogenase enzyme systems isolated from coleoptiles to be stimulated by pretreatment of the tissue with indoleacetic acid, but not by the addition of indoleacetic acid to the *in vitro* preparations. The activity of alcohol and malic dehydrogenases in *Avena* coleoptile tissue was increased respectively 200 and 150 per cent when segments of the coleoptile were treated with relatively high concentrations of indoleacetic acid (10 mg. per liter—the same concentration found most effective by Commoner and Thimann). Although not linked experimentally with studies on growth this evidence supports the view that both these dehydrogenases are closely concerned with growth, and that the hormone indoleacetic acid controls growth, at least in part, by activating them.

Employing the same high concentration of indoleacetic acid, Berger, Smith, and Avery (5) reported increases of 35 per cent or more in oxygen uptake in *Avena* coleoptile segments in sucrose solutions. But they found malate, and therefore presumably fumarate, to function as a substrate rather than as a catalyst in this reaction. Skoog (15) points out that the role of substrate rather than catalyst agrees with Lundegårdh's conclusion that fumaric and malic acids may be intermediaries but not catalysts in respiration of wheat roots.

The most extensive recent study on *Avena* coleoptile tissue (8) includes a single experiment that should be mentioned here. It concerns hormone stimulation of respiration and growth. Bonner now reports growth and respiration, as determined at the end of a 24-hour growth period, to be stimulated 40 and 38 per cent respectively in the presence of indoleacetic acid at a concentration of 10 mg. per liter.

Coleoptile tissue has also been investigated for its respiratory response to the synthetic hormone, 2,4-dichlorophenoxyacetic acid (2,4-D), and increases of 20 per cent or more in oxygen uptake in coleoptile segments

are reported by Kelly and Avery (13) for 2,4-D in concentrations ranging from somewhat less than 1 to approximately 100 mg. per liter. In the presence of malic acid the stimulation of 2,4-D is much enhanced. In similar investigations on young pea stem tissue it was found that the concentration of 2,4-D required to give a 20 per cent stimulation was of the order of 1/1000 that needed for *Avena*. Growth measurements, unfortunately, were not included in the study.

At least brief mention ought to be made of several other types of plant tissue which have also been investigated for their respiratory response to 2,4-D. For example, the carbon dioxide output of ripening pears was shown by Hansen (12) to increase as a result of dipping in 2,4-D at concentrations of from 50 to 100 mg. per liter of solution. Bean and morning-glory plants sprayed with 2,4-D (1,000 mg. per liter) showed up to 80 per cent greater carbon dioxide output than did unsprayed plants (9). Rhizomes and roots of bindweed showed an average of 70 per cent increase in the uptake of oxygen as a result of spraying intact plants with 2,4-D at 1,000 mg. per liter (16); and Southwick (17), employing various 2,4-D treatments for several varieties of peaches and one apple variety, hastened ripening and increased carbon dioxide production by 10 to 30 per cent.

One of the most recent studies (in press) involving stimulation of respiration in relation to growth on tissue other than *Avena* coleoptiles is that of Louis Nickell (14). He has kindly given me his permission to tell you about it. The tissue used was the *Rumex* virus tumor of L. M. Black, grown in culture on a fully known substrate containing no hormones. For the respiration experiments various naturally occurring and synthetic hormones were added to the Warburg vessels, among them, indoleacetic acid and 2,4-D.

Nickell observed that oxygen uptake was stimulated 40 to 60 per cent when indoleacetic acid (concentration range 0.001 to 1.0 mg. per liter) was added to the solution in the Warburg vessels. When this same hormone was added to the tissue culture substrate greatest growth (about 20 per cent over the control) was obtained at 0.01 mg. per liter. With 2,4-D the oxygen uptake was stimulated approximately 20 per cent over the control in a wide concentration range, and greatest growth in culture (about 20 per cent over the control) occurred when the synthetic hormone was added to the substrate at a concentration of 0.1 mg. per liter. It might be pointed out that although the tissue culture experi-

ments ran for three weeks, and the Warburg determination for only three hours, the stimulation range was common to both the growth and the respiration experiments.

Thus in the past decade it has been found that indoleacetic acid stimulates both respiration and growth in two widely different tissues, one of them carrying a virus disease. In one study where 2,4-D was employed, the general pattern of stimulation closely followed that for indoleacetic acid. Other studies on respiration in response to added hormone (that do not include growth) show that in living tissues certain concentrations of hormone generally stimulate respiration. There is as yet no evidence that hormones occur in tissues in concentrations that would inhibit respiration, and therefore presumably inhibit growth.

If we are to understand how hormones control metabolism, and ultimately, growth, it is important that we have further studies on the stimulation (and inhibition) of respiration in relation to growth. It is important, too, that such studies be carried out on tissues capable of giving a growth as well as respiratory response.

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Respiratory Changes in Relation to Toxicity

FREDERICK G. SMITH

Growth substances cause a variety of toxic effects in plants which often involve inhibition or alteration of respiratory metabolism. The nature of these effects is important not only as a clue to the role of growth substances in plant metabolism but also as a basis for their more successful application as herbicides. The distinction between the normal or physiological effects of growth substances and the abnormal or nonphysiological effects is not a sharp one, and for the present purpose it is useful to consider nonphysiological or toxic action in broad terms as any alteration in metabolic processes which is deleterious to plant function. We must include, then, the response to intermediate levels of growth substances ranging from those characteristic of physiological hormone action to those used in herbicide work where toxic action may become less specific and too complex for present interpretation in metabolic terms.

The object of the present discussion is to summarize the evidence relating toxicity and respiratory changes and to consider its interpretation in terms of present views of growth substance action and plant metabolism. The data are necessarily drawn from many types of experiments often not primarily designed to study toxic action and the results can be described only in broad terms. Data are confined to the effects of indoleacetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), the chief natural and artificial growth substances used in this kind of experimental work.

RESPIRATORY CHANGES IN ELONGATION, STREAMING, AND WATER UPTAKE

Much of the work on the nature of hormone action has shown a close relation between cell elongation, protoplasmic streaming, and water

uptake and respiratory activity measured by oxygen uptake or dry weight loss. Higher than optimum levels of growth substances usually, though not always, have caused parallel depression in over-all respiration and the associated physiological responses. Table 1 summarizes some of the data comparing these responses at optimum and inhibitive or toxic levels. Both IAA and 2,4-D begin to inhibit coleoptile or stem elongation and oxygen uptake in the range of 10 to 100 ppm. (13,15,18). Root elongation is much more sensitive but so far there is little if any evidence of an accompanying change in respiration (12). Protoplasmic streaming in *Avena* coleoptile has a range of sensitivity similar to elongation (33,34). In roots, however, while the optimum level for streaming is similar to that for elongation the former is strongly inhibited only at 10 ppm. or more. Active or nonosmotic water uptake and respiration in potato tuber slices (14,28) and *Avena* coleoptiles (17) are both inhibited in the 10 to 100 ppm. range. In wheat roots (25), however, 2,4-D inhibited nitrate uptake down to 0.1 ppm. with an actual increase in oxygen uptake at the upper range of 5 ppm. It appears that the correlation in stem and coleoptile between the inhibition of respiration and of other physiological effects does not hold in roots.

RESPIRATORY CHANGES IN GERMINATION

Respiratory changes have also been observed in the toxic action of growth substances in the germination and growth of whole seedlings. Here in addition to elongation, water uptake and streaming, cell division, and other physiological processes must be considered. Table 2 summarizes this work. Pratt (26) first showed that wheat-seedling growth by dry weight was inhibited beginning as low as 0.01 ppm. of IAA while oxygen uptake increased to about 50 ppm. and was strongly inhibited only at 150 ppm. This suggests that root tissue accounted for the major part of the seedling response at this stage. Hsueh and Lou (16) have reported some interesting though brief data comparing the nature of the respiratory changes in barley and rice after 2,4-D treatment. Rice, characteristically an anaerobic seed, was relatively resistant even to 1,000 ppm. of 2,4-D while barley, an aerobic seed, was completely inhibited between 140 and 700 ppm. Furthermore, there was a clear indication that carbon dioxide evolution was less inhibited than oxygen uptake. This was especially true of rice and was most marked at the two and three-day stage where control samples of barley and rice, respectively,

TABLE 1
Growth substance effects in isolated organs and tissues

PLANT MATERIAL	GROWTH SUBSTANCE (CONC. IN PPM.)		RESPIRATORY EFFECTS*	OTHER PHYSIO-LOGICAL EFFECTS*	REFERENCE
Avena coleoptile			*O_2 uptake*	*Elongation*	
	IAA	10	+50 (max.)	+25 (max.)	Commoner and Thimann (13)
		20–40	+20	−10	
	2,4-D	100	+25 (max.)		Kelly and Avery (18)
		1000	−40		
Pea stem	2,4-D	0.1–10	+45		
		100	+10		
		1000	−40		
Avena root	IAA	0.00018		+30	Bonner and Koepfli (8)
		0.018		−45	Thimann (37)
Wheat root	IAA	0.018	No change in respiration	−2	Burström (12)
		0.18		−15	
		0.8		−66	
Avena coleoptile				*Protoplasmic streaming*	
	IAA	10		+50	Sweeney (33)
		ca. 70		−30	
Avena root	IAA	0.0001–0.00001		+25 to +30	Sweeney (34)
		1		+10 to 0	
		10		−13	
			Dry weight decrease	*Water uptake*	
Potato tuber	IAA	1	+34	+34	Reinders (28)
		10	+71	+46	
		100	(became flaccid and died)		
	IAA	10		+25	Commoner, Fogel and Muller (14)
		100		−12	
			O_2 uptake		
Avena coleoptile	IAA	20	max.		Kelly (17)
		40		max.	
Wheat root	2,4-D	0.1		*Nitrate uptake* −13	Nance (25)
		1		−42	
		5	+20 to +40	−63	

*Percentage change from control; many figures estimated from graphs.

TABLE 2

Growth substance effects in seedlings

Plant Material	Growth Substance (conc. in ppm.)		Respiratory Effects*		Other Physiological Effects*	Reference
			O_2 uptake	CO_2 evolution	Dry weight change	
Wheat seedling	IAA	10	+61		−54	Pratt (26)
		50	+78		−75	
		150	−40		−75	
					Time for 50% germination	
Barley seedlings	2,4-D	70			−4	Hsueh and Lou (16)
		140			+12	
		700			no germ.	
days after treatment: (1)		1000	−67	+36		
(2)		"	−70	−6		
(3)		"	−79	−70	(control reached 100% germ.)	
Rice seedlings	2,4-D	140			−5	
		700			+20	
		1000			+25	
(3)		"	−69	+13		
(6)		"	−49	−21	(control reached 100% germ.)	
					Dry weight change	
Wheat seedling	2,4-D	5	−18	−15	−29	Taylor (36)
Mustard seedling	2,4-D	5	−25	−25		

*Percentage change from control; many figures estimated from graphs.

had reached complete germination. The suggestion was made that it is the aerobic phase of respiration which is most sensitive to 2,4-D inhibition. Taylor (36) has made a similar study of the respiratory changes following treatment of wheat and mustard seedlings but at lower levels of 2,4-D. His data also show some evidence that CO_2 evolution is less affected than oxygen uptake expecially in wheat and in the earlier stages of development. It is interesting to note further that aerobic bacteria seem to be much more sensitive to 2,4-D inhibition than anaerobic types (39). The aerobic species were inhibited from 2,000 to as low 0.2 ppm. in some cases while facultative and obligate anaerobes were unaffected or even slightly stimulated. Further comparisons of this type would be valuable, especially if organisms were selected which were more similar metabolically except for oxygen requirement.

Respiratory Changes in Larger, Intact Plants

Most work specifically on toxic or herbicidal effects of growth substances has been on larger intact plants with a single dose treatment of the aerial parts. Morphological effects vary widely with the conditions from a stunted or suppressed development to accelerated, abnormal growth. Metabolic changes may be equally profound. In general alterations in gross carbohydrate and nitrogen metabolism are found with both IAA and 2,4-D treatment at levels as low as 25 to 50 ppm. in the applied solutions. It is obviously difficult to compare such dosage levels with those in the work on isolated tissues or seedlings since the effect may be at some distance from the point of application and the amount absorbed is uncertain. However, there is characteristically a marked acceleration in hydrolysis, translocation, and utilization of the carbohydrate reserves (2,22,23,24,27,31,32) accompanied by increased protein synthesis (29,30), water content (10), and mineral content (11), and by varying degrees of cell division and enlargement. Further evidence of accelerated metabolism was shown in Luecke, Hamner, and Sell's recent report (19) of large increases in treated bean stems of the B vitamins which are coenzymes in intermediary carbohydrate metabolism. In some of these cases there was also a marked increase in respiratory activity which may have accounted for most if not all of the carbohydrate utilized (27), while in others the over-all respiratory level seemed to be unchanged (23).

A more detailed examination has been reported by the author (30)

of the changes in respiratory capacity, chemical composition, and gross histology in bean stems after treating the plant with 1,000 ppm. of 2,4-D. Within 24 hours there was a significant difference between treated and control tissues in oxygen uptake on a dry weight basis, though not on total nitrogen basis, and before any signs of abnormal meristematic activity. Maximum differences in oxygen uptake and anaerobic carbon dioxide evolution occurred by the seventh day again with the treated tissue higher on a dry weight basis and lower on a nitrogen or protein basis. Anaerobic glycolysis on a nitrogen basis showed the largest difference, with the treated slices less than one-third the control value. This difference was also reflected in the characteristically lower respiratory quotient of treated tissue. While this type of analysis gives a more complete picture of the respiratory changes following treatment it is not sufficient. For example, it does not distinguish between direct action of the growth substances on the respiratory mechanism per se and indirect effects due to changes in the available substrate. The best we can conclude at present from the toxic effects on larger intact plants is that there is a pronounced alteration in metabolism that seems to be closely associated with respiratory processes.

RESPIRATORY CHANGES IN TISSUE SLICES AND ENZYME EFFECTS

Another type of approach is found in the *in vitro* treatment of tissue slices which has the advantage of limiting the variability in the plant material and affording better control of treatment. Bean stem slices similar to those described above (30) were treated with 0.1 to 100 ppm. of 2,4-D in aerated aqueous media and the changes in oxygen uptake measured after 24 to 48 hours. No significant effects were found at 0.1 ppm.; at 1 and 10 ppm. results ranged from slight inhibition to marked acceleration but still without clear evidence of stimulated meristematic activity; and at 100 ppm. the treated slices were inhibited about 80 per cent. Mitchell, Burris, and Riker (21) have made a more extensive study of this kind in which the inhibition of oxygen uptake by 0.002 M. 2,4-D and IAA (350 and 410 ppm. respectively) in root and stem slices of several species was measured over shorter intervals. The percentage inhibition was greater in roots than in stems, except for IAA on tomato, and greater with 2,4-D than IAA, except for tomato roots. They also found that IAA of about 100 ppm. caused no change in respiratory quotient although there was 47 per cent inhibition of oxygen

uptake; and they found that the extent of inhibition was directly proportional to the oxygen tension indicating that the same systems were limited by oxygen as inhibited by IAA. They found further that the inhibition by IAA was largely reversed by washing stem slices which is similar to Audus' observation (3) that root growth inhibition by 2,4-D was similarly reversible. Further *in vitro* studies with these mature tissue slices should provide a valuable link between the classical seedling tissue studies and the herbicidal investigations with intact plants.

Finally, there have been a few investigations of the *in vivo* and *in vitro* effects of growth substances on respiratory enzymes themselves. Berger and Avery (4,5) working with *Avena* coleoptile homogenates found a marked rise in alcohol dehydrogenase activity after 15 to 24 hours treatment with 10 ppm. IAA but no effect of *in vitro* treatment until inhibitive concentrations were reached between 100 and 1,000 ppm. The Wisconsin workers (21) have failed to find any *in vitro* effect on the cytochrome, ascorbic acid, catechol, or glycolic acid oxidases by either IAA or 2,4-D in the range of about 2 to 200 ppm. The direct inhibition of oxidases or dehydrogenases seems, at present, an unlikely basis for the toxic action of growth substances.

DISCUSSION AND CONCLUSIONS

The evidence relating toxic action of growth substances with respiratory metabolism is still rather scattered and fragmentary but some interesting possibilities are apparent. In the relatively simple cases of inhibition of elongation, streaming, and water uptake in *Avena* coleoptile there seems to be a close parallel. The major evidence, in fact, for a respiratory role of plant growth substances, physiological, or nonphysiological is from studies on this classical tissue. With roots the evidence is limited, though it looks as if the inhibition of cell elongation and water uptake may occur at concentrations of growth substances which do not affect over-all respiration. This, of course, should not be considered evidence against any connection with respiratory metabolism since the fraction of total respiration involved in these processes may be small (13) or may not be expressed in terms of gross oxygen uptake or dry weight change. Our greatest need is clearly for a closer examination of the metabolic changes which accompany growth substance treatment.

In the case of intact plants, seedlings or larger, toxic effects are more varied and complex, and there is no close parallel between respiratory

inhibition and the other physiological effects. Toxic action in more mature plants may actually result in increased total respiration, with or without accelerated growth and development. Probably the fundamental effect, however, is the tendency to a change in type of respiration.

What evidence do we have from these toxic effects of the way growth substances affect respiratory metabolism? One of the most interesting possibilities is the greater sensitivity of the aerobic than of the anaerobic phase of respiration in seedlings to 2,4-D inhibition. This may also be involved in the differing sensitivities of roots and shoots if Taylor's observations on rice and wheat (35) on the relative abilities of these organs to grow anaerobically are of general occurrence. Mitchell, Burris, and Riker's (21) failure to find any change in respiratory quotient in bean stem tissue after short term *in vitro* treatment may indicate only that this aerobic-anaerobic difference is confined to less mature tissues. There is, in fact, considerable evidence for a change in respiratory mechanism from seedling to mature leaf tissue (1,20). It would be interesting to know as well whether the difference in monocot and dicot sensitivity to 2,4-D at emergence might be explainable in these terms. The special sensitivity of aerobic phases of respiration to growth substance inhibition is at least consistent with the fact that plant growth in general is aerobic, and with the present views of Thimann, Bonner, and others (7,38) that IAA affects growth through a sulfhydryl enzyme system and is involved in aerobic phosphate transfer.

In the light of recent work of Thimann and W. D. Bonner (38) and J. Bonner (7) it is interesting to compare the action of known respiratory inhibitors with that of the growth substances, themselves. At appropriate concentrations most of the respiratory inhibitors like the growth substances will either accelerate or inhibit growth. In some cases the concentrations for growth inhibition and respiratory inhibition are similar, for example, the action of cyanide and IAA on *Avena* coleoptile (6). In other cases growth may be more sensitive than respiration, as in iodoacetate (13) and fluoride action (9) and the effects of growth substances on elongation and water uptake in roots. Furthermore, at a given concentration growth may be inhibited and respiration stimulated as in the case of 2,4-dinitrophenol (7) and in some types of 2,4-D treatment. There are also some parallel changes in the inhibitive effects of respiratory inhibitors and growth substances with the age of tissues (38). These comparisons are not meant to imply that IAA or 2,4-D act necessarily by the

same mechanism as any of the respiratory inhibitors but only to indicate that the various types of growth and respiratory effects shown by the growth substances have parallels in the action of metabolic inhibitors whose action is more or less well known. This is merely a further justification for the useful working principle that growth substances act, either in a physiological or in a toxic way, by some effect on respiratory metabolism.

In conclusion, we are likely to make more progress toward understanding the mechanism of growth substance action when we have a more complete knowledge of the respiratory machinery of the tissues in which the action of growth substances is studied, or possibly when we are able to study their action in tissues whose respiratory systems are already better known.

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Tissue Responses
to Growth Substances

Electrical Polarity and Auxins

A. R. SCHRANK

G ROWTH curvature responses of plants are dependent on an integrating mechanism which permits the individual cells of a system to operate collectively as a coordinated unit. In the absence of a highly specialized nervous system in plants some other more primitive device must be functioning. Evidence has been presented to show that living organisms are surrounded and permeated by electrical fields which are generated by the polar components of the system (17). These fields have been looked upon as an electrical correlation mechanism, which operates by transferring electrical energy within the field. In the simplest aspect the flow of current could be accomplished by the movement of ions or other electrically charged particles. This, however, should not be taken to imply that the transfer of electrically charged particles is necessarily accomplished by electrophoresis.

A prodigious mass of experimental data (3,37), which has been accumulated over a period of years, serves as the basis for the far-reaching Cholodny-Went (9) theory of plant growth responses. More recent evidence also continues to support the postulation that growth curvatures are due to an unequal distribution of auxin in the opposite sides of the curving organ (21,22,38). Although the detailed explanation of precisely how the uneven distribution is accomplished is not always apparent, it is generally accepted that the lateral transport of auxin is a rather significant intermediate link in many of the curvature responses. It is obvious that auxins cannot be transported of themselves, and that some additional mechanism in the form of an oriented force is essential. A challenging hypothesis can be formulated by assuming that the inherent electrical fields function as the required directing forces, and that auxin is thereby transported in the form of electrically charged particles.

In many studies of auxin-controlled curvatures the *Avena* coleoptile has been used as the standard experimental material. Therefore, this discussion is to analyze the inherent electrical pattern, as a possible correlation mechanism, of the same plant. For convenience of organization this paper will be divided into the three following parts: examination of the bioelectrical pattern of the nonstimulated coleoptile; changes of certain components of the electrical field that result from stimulation by different types of energy; effects of altering the inherent electrical field by superimposing an electrical polarity of external origin.

The Electrical Field of the Nonstimulated Coleoptile

By performing a large number of experiments in which every possible precaution was taken to keep all of the environmental factors constant, Wilks was able to work out the distribution of electrical potentials of the *Avena* coleoptile in considerable detail (39). His observations were completely confirmed and extended by Schrank during the course of a later investigation (27). The results of these many experiments can be briefly summarized by the use of a diagram, which shows the dimensions of the electrical pattern quite distinctly. In presenting the diagram, shown in Figure 1, a few explanatory remarks should be included. First, it is important to remember that one of the general characteristics of electrical polarities of living systems is that they vary quite appreciably from one instant to the next, even though a source of stimulation is not apparent (39). It is not uncommon for the longitudinal electrical polarity of an intact *Avena* coleoptile to change from 10 to 20 millivolts during the course of ten minutes. Second, the magnitude of a given polarity is not necessarily the same in all plants which are otherwise similar. These circumstances naturally demand that the magnitudes of the voltages expressed in the figure are only approximate averages.

Examination of Figure 1 reveals several relevant points to which attention should be directed. In so far as the external longitudinal electrical polarity is concerned (circuits labeled X, Y, and Z), it becomes apparent that the apical region is electronegative to the base; the magnitude of the polarity is of the order of about 50 millivolts. The most negative region, with respect to the base, is the section about 5 to 8 millimeters below the apex. This also appears to be the region of the greatest radial polarity (circuits L, M, N, O, and P). It is noted that the orientation of the radial polarities of the apical 17 millimeters is such

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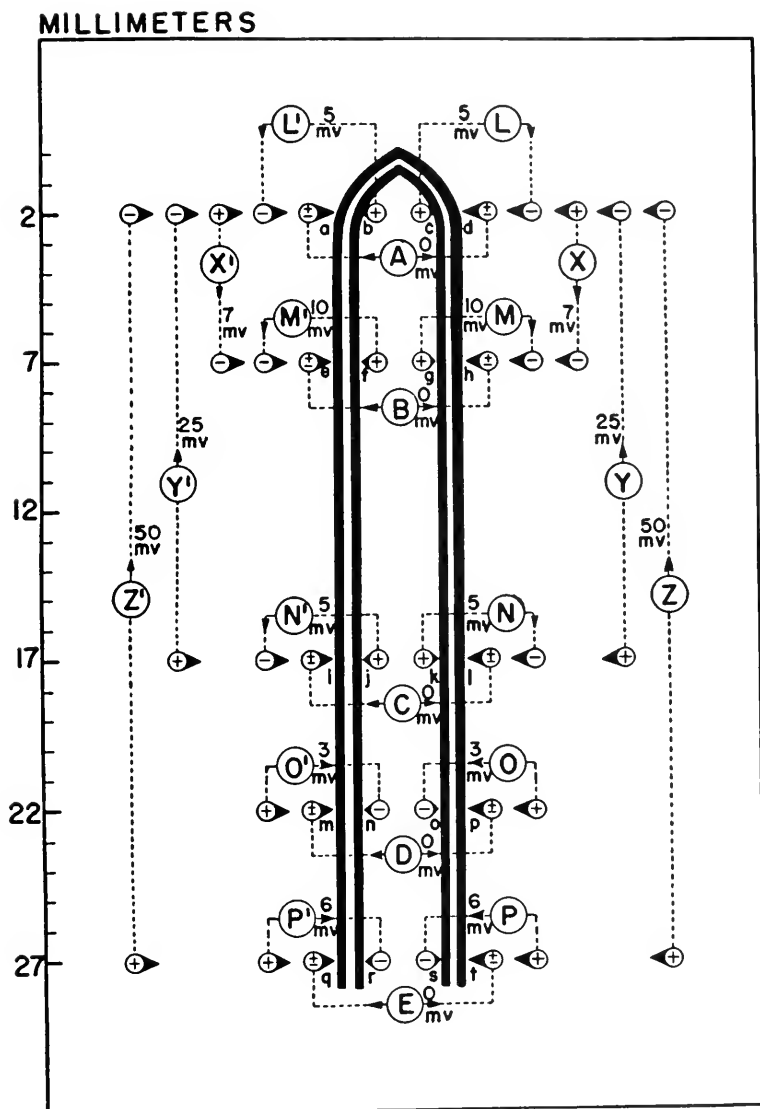
Figure 1. Diagram showing some of the components of the electrical field of the *Avena* coleoptile under constant experimental conditions. Arrows in the external circuits indicate the direction of current flow.

that the inside is electropositive to the outside. In the more basal regions the radial polarity is reversed. This would indicate that for the internal longitudinal polarity the basal region is electronegative to the apex (27,39). Not all of the data in the literature are in agreement on this point (12). Finally, Figure 1 shows that no transverse polarity (circuits A, B, C, D, and E) is manifested by the nonstimulated coleoptile when it is kept in the vertical position.

The possibility of a definite system of electrical currents within the coleoptile becomes apparent from the figure. The implication is that the electromotive forces of the cells would supply the energy required for continuous cell correlation. This concept of electrical correlation naturally assumes that some cells of the system are capable of absorbing the electrical energy that is generated by other cells some distance away. Whether or not coleoptile cells can absorb electrical energy will be indicated by experiments included later in this paper.

One additional statement about the direction of auxin movement must be made at this time. It is known that the transport is from the apex toward the base in the coleoptile in the vertical position. In terms of the external longitudinal electrical polarity this means that the transport is toward the electropositive region of the system.

TRANSVERSE ELECTRICAL RESPONSES TO VARIOUS TYPES OF STIMULATION

Stimulation by gravity.—The effect of stimulation by gravity on the electrical field of the *Avena* coleoptile has been investigated extensively by Schrank (23,27). His data disclose a number of changes in the various electrical polarities when a plant is shifted from the vertical to the horizontal position. Of these several changes only the transverse component will be included in the present treatment. Figure 2 was selected to show the typical results. Figure 2A shows that a transverse electrical polarity in the apical portion of the coleoptile (contacts 2 millimeters below the apex) is nonexistent as long as the seedling remains in the upright position. When it is placed in the horizontal position the under side becomes positive to the upper side; even the first reading, which was taken one minute after the change of position, indicates the beginning of the polarity. It is worth while to note that this transverse electrical change occurs all along the longitudinal axis of the coleoptile, but smaller potential differences are established in the more basal regions

(27). Similar electrical changes have also been observed by other workers for stems of other plants (2,4,5). However, Brauner and others reported comparable results from plants that had been killed in boiling water and from models constructed out of nonliving membranes (6,7,8). Because of the results obtained from the models these workers were led to maintain that the electrical responses were nonliving phenomena, and Brauner and Amlong ascribed the geoelectrical changes to streaming potentials (7). The data shown in Figure 2 were obtained from non-
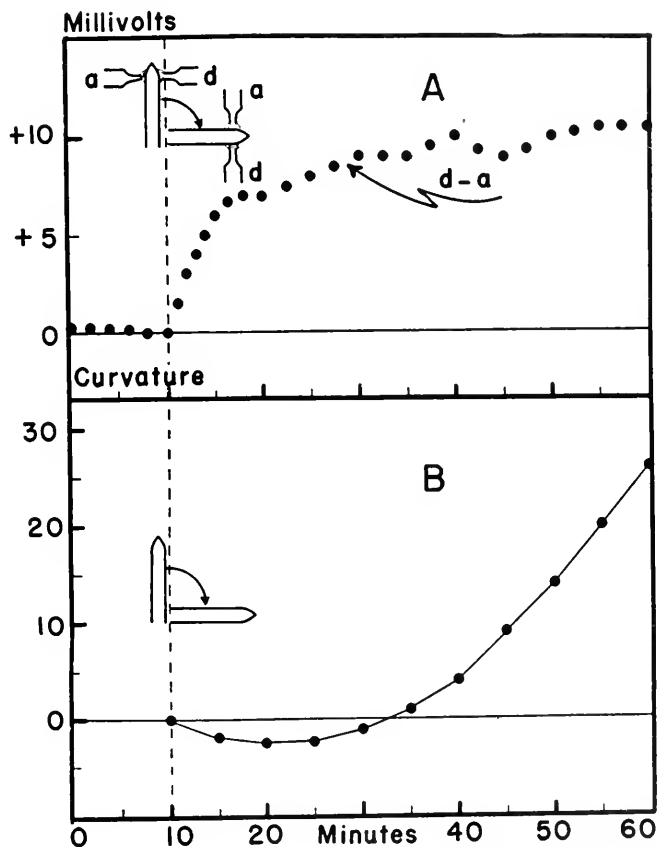
Figure 2. *A.* Transverse electrical changes in *Avena* coleoptiles that are rotated from vertical to horizontal position. Contacts 2 millimeters below apex. Average of 6 experiments. *B.* Upward curvature of coleoptiles in horizontal position measured in ocular scale divisions of 18 per millimeter. Average of 10 experiments.

injured living tissues exhibiting normal growth and bending responses (27). Coleoptiles, which were immersed in boiling water for 15 seconds, did not show any electrical changes when they were placed in the horizontal position. Further control experiments also disclosed that the electrical responses were not due to electrode phenomena, which might have been the case in earlier work.

The curvature induced by gravity, represented by movement of the coleoptile tip measured in ocular scale divisions (18 per millimeter), is shown in Figure 2B. This curve indicates that upward bending starts only after the plant has been in the horizontal position for 22 minutes. When the relationship between the known velocity of transport (19, 36,37) and the minimum distance that the auxin has to be displaced in lateral redistribution is evaluated, it is at once apparent that the transverse electrical polarity in *Avena* is established before an unequal distribution of auxin is considered possible (23). This sequence of events also indicates that the electrical polarity in the coleoptile is not dependent on the metabolic process for which the auxin is directly responsible. These results and inferences permit the arrangement of coleoptile responses to gravity in the following order: establishment of a transverse electrical polarity in which the underside becomes electropositive; unequal distribution of auxins (in this instance the auxin is again transported toward the electropositive portion of the plant); and upward curvature.

Stimulation by light.—Only a limited number of investigators have been concerned with the various phases of the effects of incandescent light on the electrical potentials of etiolated seedlings (11,26,35). When the field is narrowed to the effect of unilateral illumination on the transverse electrical polarity of the *Avena* coleoptile the only information that is available comes from the preliminary experiments of Schrank (25) and Oppenoorth (20). Figure 3A shows the typical electrical response of the most apical cells of an isolated *Avena* coleoptile (contacts 0.5 millimeter below the apex), which was illuminated continuously by a light intensity of 16 foot-candles at the coleoptile position. As indicated by the curve in Figure 3A, after ten minutes of illumination the shaded side becomes electronegative to the lighted side. This first negative variation was observed in about 65 per cent of the experiments. Later the shaded side always becomes electropositive to the lighted side, reaching an average maximum of 8.2 millivolts. The corresponding bend-

ing toward the light expressed as horizontal tip movement is shown in Figure 3B. As in the previous experiments the final transverse electrical polarity is again established before the curvature starts. The orientation of the electrical polarity with respect to the subsequent bending is the same as it was when the plants were stimulated by gravity.

These observations are beset by several limitations. The indicated electrical changes have been observed only for the most apical cells of the isolated sheath. Whether or not unilateral illumination induces a transverse electrical polarity along the entire longitudinal axis is not known. Furthermore, the quantity of light that was used in these
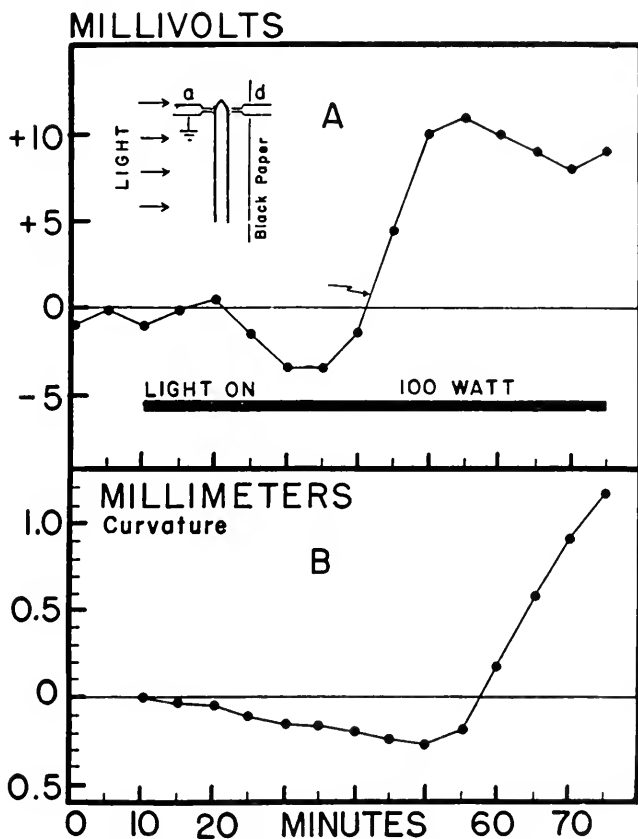
Figure 3. *A*. Effect of continuous unilateral illumination of an isolated *Avena* sheath on its transverse electrical polarity 0.5 millimeter below the apex. *B*. Phototropic bending of the same coleoptile.

experiments was much greater than the amount required to give the first maximum positive curvature. In this instance the implication is that photoinactivation of auxin may have been an unusually prominent factor in the induction of the curvature. Finally, the data in Figure 3A are not in apparent agreement with the results obtained by Oppenoorth (20), who found that unilateral illumination of the coleoptile by 500 meter-candle-seconds of unfiltered mercury light caused the lighted side to become electropositive to the shaded side. He obtained this polarity by subtracting the longitudinal electrical changes of the shaded side from the simultaneous changes of the lighted side. A common basal contact was used in measuring these electrical responses.

Mechanical stimulation.—Growth curvature responses to mechanical stimulation, which were first studied in tendrils, are also prevalent in etiolated seedlings. Years ago Stark (33) demonstrated that he could induce bending of the coleoptile by stroking it on one side with a cork rod. These observations, along with the frequently reported fact that mechanical stimulation of a segment of living tissue causes it to become electronegative to the unstimulated portion (16,18), led Schrank (24) to investigate the relationship between the electrical and curvature responses of the *Avena* coleoptile to mechanical stimulation. Preliminary experiments confirmed Stark's observation that the coleoptile would bend toward the side that was lightly tapped and demonstrated that a transverse electrical polarity was established with the stimulated side becoming electronegative to the opposite side. In subsequent experiments mechanical stimuli were applied by the use of an electrically operated vibrator, which was mounted on a micromanipulator in order that the position of the stimulating device could be accurately duplicated.

Curves in Figure 4A show the average magnitude of the transverse electrical polarity that is established in the apical region of *Avena* when the apical 10 millimeters of one side are mechanically stimulated by the vibrator for the duration indicated. The magnitude of the polarity and the rate of its decrease are dependent on the duration of the stimulation. In Figure 4B the corresponding curvatures are shown. The magnitude and rate of bending are also dependent on the duration of the stimulation with the direction of bending being toward the negative side of the plant. Since mechanical stimuli cannot be applied to the coleoptile without bending or displacing it from its original position (the reason for starting the curves below the zero line), there is no way to determine exactly

when the curvature starts toward the stimulated side. (A good guess would be that curvature due to growth starts as the coleoptile tip passes its prestimulation position.) Thus it is impossible to state in this situation whether or not the electrical polarity is established before the curvature starts. Logically it would seem that it is, because the rate and magnitude of the curvature can be approximately predicted from the magnitude of the electrical response and its rate of change.
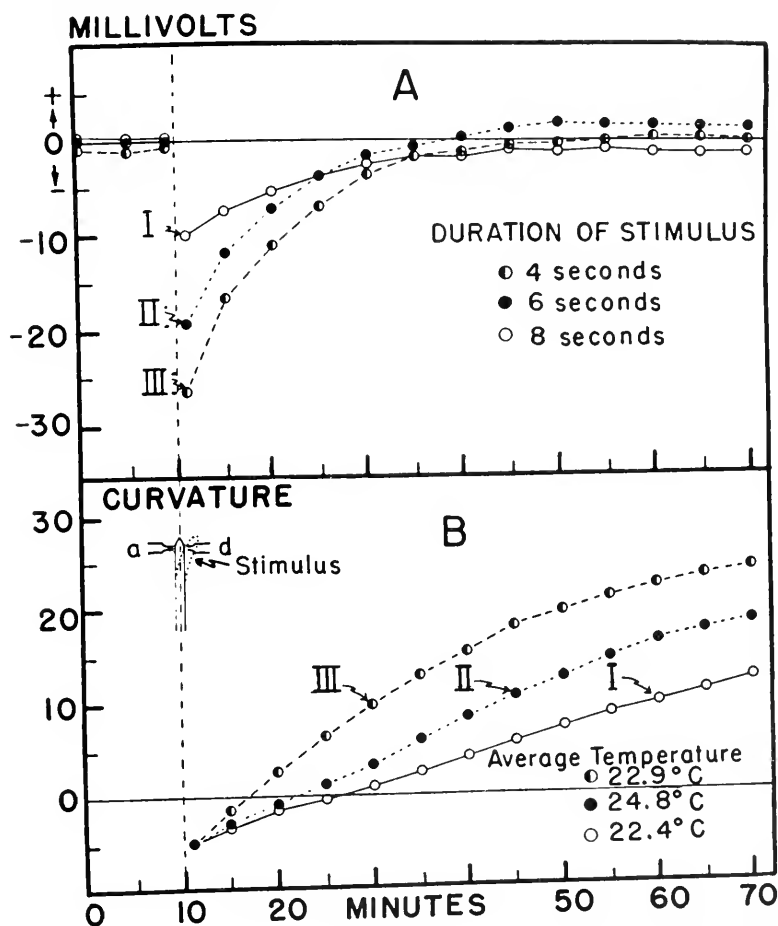
Figure 4. *A*. Transverse electrical polarities established by *Avena* coleoptiles 1.67 millimeters below the apex in response to mechanical stimulation of one side of the apical 10 millimeters. Each curve is an average of 10 experiments. *B*. Corresponding average curvatures in scale divisions of 18 per millimeter.

Without arrogating the space to review the specific data, two other implications of these experiments should be contemplated. It was previously noted that when the coleoptile is placed in the horizontal position the under side becomes positive to the upper. Simultaneous mechanical stimulation of the apical 10 millimeters of the under side establishes a reversed electrical polarity (opposite to the polarity induced by gravity alone), inhibits upward curvature for 25 minutes, and decreases the subsequent rate of bending. Geotropic bending can be entirely prevented by repeated application of mechanical stimuli to the under side at five-minute intervals. This inhibition apparently is not due to an injury phenomenon because similar stimulation of the upper side does not inhibit upward curvature. Stimulation of the upper side results in an electrical polarity larger than that obtained from gravity alone.

Indications are that the curvature observed in these experiments is due to growth. Coleoptiles that have had the apical 3 millimeters removed 2 hours and 10 minutes before stimulation cannot be induced to bend by a combination of mechanical and gravitational stimuli. This is taken to verify the necessity of the presence of auxin for the curvature responses that were previously observed. The fact that the electrical polarity established by these plants is similar to that in nondecapitated coleoptiles has been interpreted to indicate further that the auxin-controlled mechanism is not required for the generation of electrical polarities of this nature.

In the series of experiments that has just been reviewed it was demonstrated that the *Avena* coleoptile responds to stimulation by gravity, light, or mechanical means by establishing a transverse electrical polarity. These stimuli also induce bending. In all of these instances the polarity is established before the bending starts, and the direction of bending is such that the electropositive side always becomes the convex side. If it is assumed that the lateral transport of auxin is the fundamental intermediate link in these curvature responses, then it follows that the auxin is invariably transported toward the electropositive side of the plant. This implies that the auxin is transported as a negatively charged particle. These data are considered to be compatible with the Went-Kögl electrophoretic transport theory. However, extreme caution should be exercised in drawing categorical and far-reaching conclusions about the causal relationship between the electrical polarity and the auxin transport.

Effects of Applied Current on
Electrical Pattern and Curvature

Perhaps the most direct way to alter the electrical field of a living organism is to superimpose a field or polarity of external origin. This approach has been taken by several investigators. Wilks (39), Clark (12,13), duBuy and Olson (14), Cholodny and Sankewitsch (10), and Kögl (15) have all studied the various aspects of applied direct current on growth and auxin transport in the *Avena* coleoptile. Some of their reports (10,15,39) indicate that the effects of applied current on growth responses are dependent on the polarity of the current, but one maintains that the curvature obtained is not dependent on the polarity of stimulation (14). Since the publication of these observations an additional series of papers has appeared in which the effects of applied current on the electrical polarity and curvature of the coleoptile were observed simultaneously. In the experiments to be examined at present the current was applied either transversely or longitudinally.

Current applied transversely.—When 5 to 20 microamperes of direct current are applied transversely for 2 to 10 minutes the coleoptile always responds by establishing a transverse electrical polarity (28). (Control experiments show that such electrical responses are obtained only from living tissue.) In Figure 5 a group of average curves are shown, which represent the transverse electrical polarities induced by applying a given current (10 microamperes for 10 minutes) at various distances below the apex. These curves show that a given quantity of current induces the maximum electrical polarity when it is applied to levels more than 5 millimeters below the apex. The fact that these electrical polarities are established is taken to indicate that the applied current did flow through the living tissue, a point not always clear in previous papers. Also current applied transversely in the manner just described always causes the coleoptile to bend. Figure 6 presents a diagrammatic account of this bending process when 10 microamperes were applied for 10 minutes at a level 10 millimeters basal to the apex. The initial curvature, in and above the contact region, is toward the positive pole of the current-applying circuit; however, there is a subsequent bending, basal to the contact region, in the opposite direction. The maximum angular curvatures that were observed in the initial direction, resulting from current (10 microamperes for 10 minutes) applied transversely to various levels below the apex, are shown in Table 1.
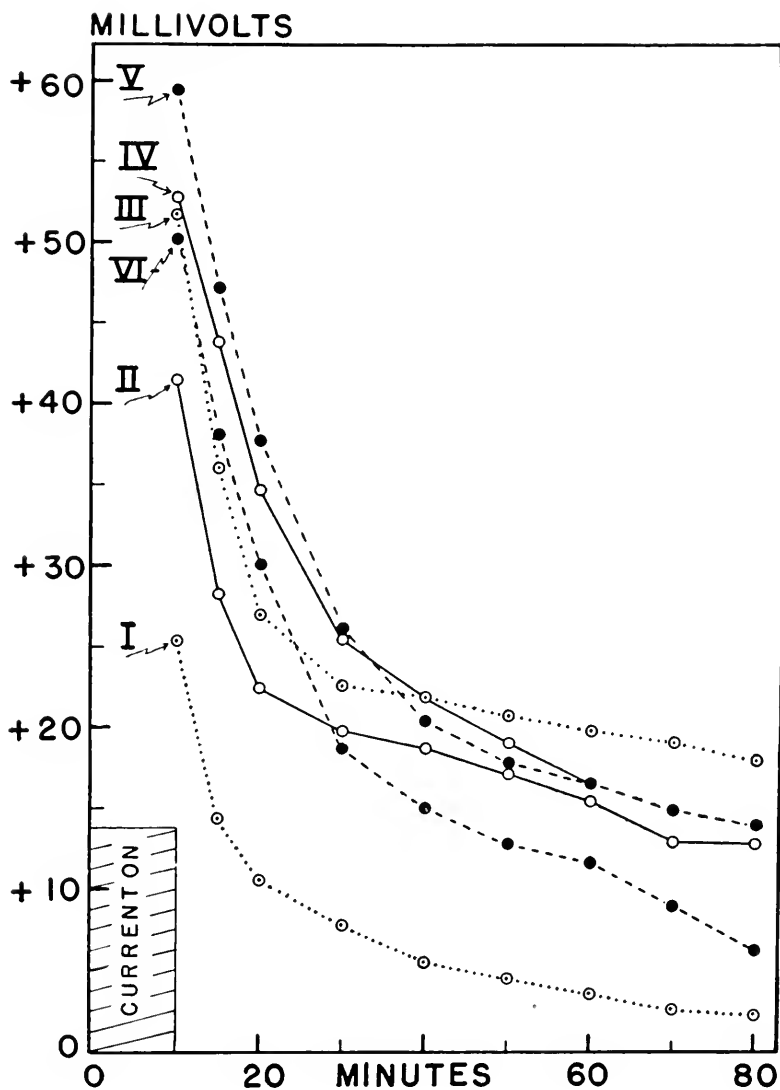
Figure 5. Electrical polarities induced by applying 10 microamperes for 10 minutes transversely to the *Avena* coleoptile at 2, 5, 10, 15, 20, and 25 millimeters below the apex. Each curve is an average of 10 or more experiments and numbered beginning with the most apical level.
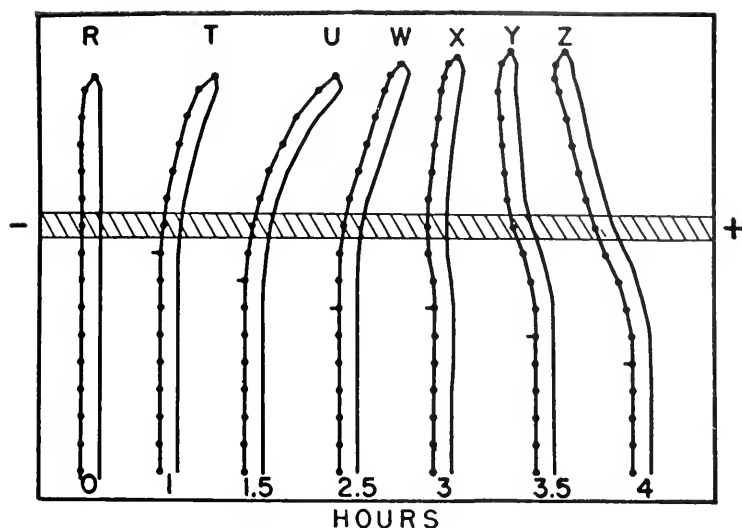
Figure 6. Successive stages of *Avena* coleoptile curvature after transverse application of 10 microamperes for 10 minutes. Current applied 10 millimeters below the apex.

These curvature data permit several interesting observations. First, it would appear that the applied current is inducing the curvature by affecting the auxin-controlled mechanisms. This tentative conclusion is drawn from the fact that the zone of curvature moves toward the base, which is clearly shown in Figure 6. More specifically, this could be interpreted to mean that the current has actually affected the lateral transport of the auxin. Secondly, from Thimann's work on the distribution of auxin along the longitudinal axis of the coleoptile (34) and the

TABLE 1

Degrees of curvature of the *Avena* coleoptile

| | CONTACTS MILLIMETERS BELOW APEX | | | | EXPERIMENT NUMBER |
	5	10	15	20	
	15	8	4	2	1
Curvature	10	6	6	5	2
in	13	12	6	3	3
degrees	10	6	3	2	4
	11	9	7	4	5
Average	11.8	8.2	5.2	3.2	

data shown in Table 1, it appears even more likely that the hormone mechanism is involved in the bending induced by current. Finally, it has been demonstrated that phototropic bending can be inhibited by transversely applied current, and that curvature responses to electrical and light stimulation are apparently limited by a common factor (29). The premise that the responses to both types of stimulation are controlled by auxin would explain this observation.

Perhaps the most striking fact is that the initial curvature is toward the electropositive side of the plant and not toward the negative side as it was in all of the previous instances. Several implications must be considered in the evaluation of this fact. What is needed most are additional experiments which would expose the details of the pathways of current flow through the tissue. This might help to visualize how bending is induced apically to the contact region, as well as to help account for the direction of the initial bending. Another point to be kept in mind is that the electrical polarity measured in these experiments, in contrast to the previous ones, very likely is due to polarization phenomena rather than changes in the inherent electrical pattern. This inference is made only on an indirect basis from the work of Berry *et al.* (1) on the onion root.

Current applied longitudinally.—DuBuy and Olson (14) were the first to report that the *Avena* coleoptile could be made to bend by applying direct current longitudinally to one side of the apical 5 millimeters. The curvature, which they observed, was always toward the side on which the contacts were placed and, according to them, not dependent on the direction of the current flow. When these experiments were duplicated and extended, several additional facts were disclosed (30). Figure 7 presents some of these observations. Ten microamperes applied longitudinally to one side of the apical 5 millimeters (polarity indicated in the insets) cause marked changes in the longitudinal polarity of the coleoptile. Current flowing from the apex toward the base reverses the inherent electrical polarity and gives the response shown by the average curve I in Figure 7A. Effects of the same current flowing in the opposite direction are shown by curve II, which is quite obviously different from curve I. Current flowing from the apex toward the base enhances rather than reverses the inherent electrical polarity. The magnitude of the initial response is not as large as in curve I, and the sequence of events
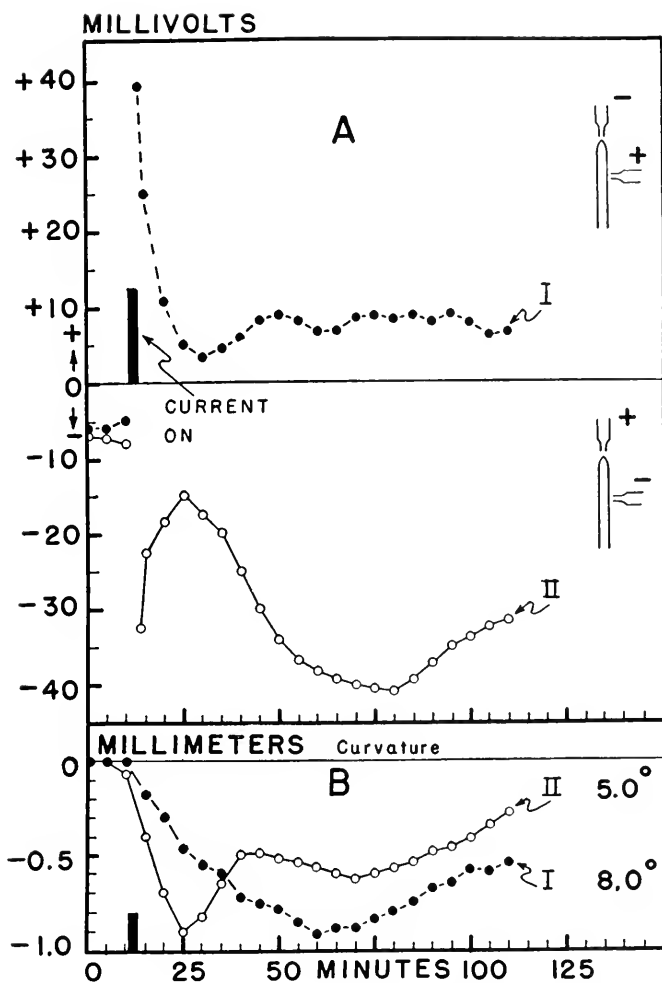
Figure 7. *A*. Electrical responses of *Avena* coleoptiles to 10 microamperes of direct current applied longitudinally for 2 minutes to a 5 millimeter apical segment. Each curve is an average of 5 or more experiments. *B*. Corresponding average curvatures plotted as millimeters of horizontal tip movement. Numerals at the right of the curves show the average angular curvatures at the end of the experiments.

is distinctly different. These facts definitely show that the polarity of the applied current has an effect on the electrical response.

In Figure 7B the corresponding curvatures are represented by graphs of the movement of the apex and by numerals indicating the angular curvature at the end of the experiments. The direction of bending is always toward the side on which the contacts were placed, thus confirming, in this part, the observations of duBuy and Olson (14). However, curves I and II in Figure 7B demonstrate that a given current flowing from the base toward the apex through the coleoptile induces curvature more effectively than the same current flowing in the opposite direction. On the basis of known effects of current on elongation (10,39) this can be interpreted to indicate that current flowing toward the apex inhibits elongation more effectively than the same current flowing in the reversed direction. These observations, in contradiction to the results previously published by duBuy and Olson (14), show that the magnitude and the temporal sequence of the curvature are dependent on the polarity of the applied current. This point very likely will assume considerable importance in the final and complete explanation of how current affects growth.

Finally, one additional group of experiments will be introduced. It has recently been shown that direct current, (10 microamperes for 2 minutes) applied in the manner just described, always inhibits bending toward 200 meter-candle-seconds of unilateral illumination (31). The extent of the curvature inhibition and the temporal sequence of events are again dependent on the polarity of the applied current and on the position of the contacts with respect to the light source. Results of these experiments show that current applied longitudinally on the lighted side inhibits rather than accelerates curvature toward the light, while current applied to the shaded side inhibits bending toward the light somewhat more effectively. The polarity of the applied current which is most effective on the lighted side is least effective on the shaded side.

The results of these last experiments seem to indicate that longitudinally applied current brings about the growth curvature by influencing the auxin-controlled mechanism, but it is rather difficult to explain, on the basis of the available evidence, precisely how applied current affects growth. It has been observed that current applied in this manner reversibly inhibits protoplasmic streaming (14). If the transport of auxin is dependent on the streaming, it follows that current could have

some of its effects by inhibiting the movement of the active auxin (32). This explanation, however, is inadequate to account for the inhibition of the phototropic bending by current applied longitudinally to the illuminated side. Furthermore, the data that are available (14) indicate that the inhibition of streaming is independent of the polarity of the applied current, while many of the experiments which have been reviewed in this paper show that the magnitude and temporal sequence of the curvature of the coleoptile in response to current are dependent on both the polarity and strength of the electrical stimulation. It is obvious that protoplasmic streaming, at best, can play only a limited role in these electrically induced growth phenomena.

A number of experimental observations that have been presented would permit the possibility of accepting the inherent electrical field as the primary integrating mechanism of the *Avena* coleoptile. 1) In stimulation by light, gravity, or mechanical means the electrical polarity changes apparently precede the hormone redistribution and curvature. 2) The orientation of the transverse polarity with reference to the direction of the subsequent bending is consistent in all of these instances. 3) Experimental evidence has been presented to show that the inherent electrical field of the coleoptile is not dependent on the same auxin-controlled process that is required for elongation. 4) Cells of the coleoptile are capable of absorbing electrical energy; this is demonstrated by the fact that curvature can be induced by either transversely or longitudinally applied current. These growth responses in every instance are dependent on the polarity of the applied current. 5) Additional evidence indicates that applied current induces its effect on growth via the same mechanism that unilateral illumination does; at least both are limited by a common factor.

Obviously the summarized facts are still inadequate to prove conclusively that the inherent electrical field functions as the primary correlating mechanism in the *Avena* coleoptile. It is now certain that an externally imposed electrical field does affect the growth of the coleoptile in a polar fashion, but much more information is needed to clarify the details of this relationship. Additional data are also necessary to account for the fact that the coleoptile bends toward the electropositive pole of the current-applying circuit, while in the remainder of the tropisms the curvature is away from the positive side of the plant.

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Translocation of Growth-Regulating Substances and Their Effect on Tissue Composition

JOHN W. MITCHELL

PLANT growth regulating substances are readily absorbed by plants. These chemicals penetrate the living surface cells of most plant parts and seem to move as readily through intact epidermal cells as through injured ones. Synthetic growth regulators readily gain entrance to the plant when applied to roots, stems, leaves, flowers, or fruits. They are absorbed by these organs even though the surface cells of some may be protected by thick walls and a layer of cutin.

Three methods have been used in studying the rate of absorption of growth-regulating substances: 1) the detection of morphological or histological responses that occur some distance from the treated area has been used to indicate that the plant has absorbed the growth-regulating substance; 2) measurement of the amount of residual growth regulator left on the surface of a treated area has been used to indicate the amount of the chemical absorbed; 3) radioactive tracers have been used to some extent in studying the absorption of these chemicals. As yet, however, we do not have a direct and completely reliable method of measuring the absorption of growth regulators by plants. All three methods have drawbacks. If we attempt to measure absorption by means of radioactivity, or by evaluating a morphological or histological response that occurs some distance from the treated area, then translocation of the chemical is involved as well as its absorption. If we attempt to measure absorption by detecting the amount of the chemical remaining on the treated surface of the plant, then we are confronted with the problem of how to remove this surface residue quantitatively so that it can be measured accurately. In spite of these difficulties some reliable

results have been obtained regarding the absorption of plant growth regulators.

Such substances as indoleacetic acid and the phenoxy compounds are apparently absorbed by most leaf-surface cells of plants. Their absorption by leaves does not seem to be related to the presence of stomatal openings (59).

Because of the inadequacy of methods at hand we have not yet been able to measure directly the effect of age or stage of development on the rate of absorption of growth regulators by plants. It is known that when older, more mature leaves or stems are treated the plants do not respond as readily to growth-regulating substances as when the chemicals are applied to young vigorously growing parts, but this may be due to factors other than absorption of the chemical (59).

The evidence, so far, indicates that leaves of certain dicotyledonous plants may absorb certain growth-regulating substances somewhat more readily than do leaves of monocots such as oats and barley (62). Thus when 10 micrograms of radioactive 2-iodo-3-nitrobenzoic acid was applied the young leaves of bean plants absorbed roughly twice the amount absorbed by the leaves of barley plants. Thus far, most of the work concerning the absorption of growth regulators by plants has not dealt with the direct measurement of the rate of absorption of the chemical. However, Rice (48), using a direct method of measurement, reports that a large part of the 2,4-dichlorophenoxyacetic acid that he placed on bean leaves was absorbed during the first 4 hours following treatment. He measured the amount of the chemical that was left on the surface of the leaf then subtracted this amount from that which had been initially added thus obtaining the rate of absorption.

Bean plants have absorbed a sufficient amount of 2,4-D within 4 to 6 hours to bring about a maximum response (60). Absorption of 2,4-D apparently begins as soon as the chemical comes in contact with the plant. In order for activated charcoal to absorb effectively 2,4-D that had been added to the surface of a plant, Weaver (57) had to apply the charcoal to the surface within 15 minutes after the chemical came into contact with the plant. It is a matter of common observation that young and succulent plants often show marked growth responses within 30 to 60 minutes following the application of a growth regulator of the phenoxy type, and it has long been known that indoleacetic acid is quickly absorbed by succulent tissues of such plants as tomato, bean,

and oat coleoptiles (22,35,61). The rate of absorption of growth-regulating substances is rapid, on the basis of these data, but the absorption of these chemicals is influenced to some extent by environmental factors, such as light, temperature, and the presence of a wetting agent or of surface-active substances. That light influences the rate of absorption of 2,4-D was shown by Rice (48). Absorption of the ammonium salt of 2,4-D by bean leaves was greatest immediately after the chemical was applied. More of the salt was absorbed by illuminated leaves than by others kept in darkness. Leaves do, however, absorb 2,4-D at a slower rate during periods of darkness. Rice reported that increasing the light intensity from 100 to 900 foot-candles had no appreciable effect on the rate of absorption of the ammonium salt of 2,4-D. Temperature, on the other hand, affected absorption of the salt since the amount taken up by a plant at 46° to 58°F, was less than at a temperature of 78° to 80°F. Injurious effects of 2,4-D are more pronounced in plants grown at relatively high temperatures (70°–85°F.) than in plants grown at relatively low temperatures (5°–15°F.) (23,27).

Wetting agents and some hygroscopic substances that dissolve growth-regulating chemicals have a marked effect upon the activity of the growth regulator. This is apparently so because they make it possible for the plant to absorb the growth-regulating substances more readily than when the wetting agents are not added to the aqueous mixture. Polyethylene glycols are among these activating substances (64). Growth-regulating chemicals are generally soluble in polyethylene glycols. Some of the glycols are hygroscopic. They serve as solvents and wetting agents and tend to keep the growth-modifying substance in close contact with the surface of the plant (12,37). It is reasoned that growth regulators applied as aqueous mixtures of the salts, such as the sodium or ammonium salt of 2,4-D, tend to crystallize out as the water carrier evaporates. Absorption of the growth regulator is thus reduced since upon crystallization the chemical is no longer in close contact with the surface of the plant. By actual measurement the rate of absorption of ammonium salt of 2,4-D in water by bean leaves decreased after the first 4 hours. When Carbowax was added to the mixture of the salt and water, a relatively rapid rate of absorption prevailed for an extended length of time (11).

In a similar way 2,4-D in a dust carrier was relatively ineffective as an herbicide, but when 3 per cent of Carbowax 1500 was added, a 0.05

per cent concentration was sufficient to kill morning glory plants (28). With respect to the mode of action of Carbowax 1500, its hygroscopic properties are probably not of prime importance, since glycerine and Carbowax both increased the effectiveness of 2,4-D in a dust carrier, while calcium chloride had no effect in this respect. Dispersing agents, such as Tween 20 and Emulfors, and surface-active substances, such as soapless washing powders which contain lauryl sulphates, are effective in increasing the activity of the acid and salt forms of 2,4-D (30,54).

The nature of the action of surface-active substances is not understood fully. Their effectiveness may be due in part to the fact that they serve to spread the growth regulating chemical and make it adhere closely to the surface of the plant.

Staniforth and Loomis (54) concluded from experiments with corn, flax, and soybeans that the power of these detergents to reduce surface tension is not directly responsible for their effect on the activity of aqueous 2,4-D mixtures. Five hundredths of one per cent of a detergent gave a maximum reduction in surface tension, but the detergent increased the effectiveness of 2,4-D up to a concentration of 2 per cent of the surface-active substance. It is of further interest that these detergents may increase the effectiveness of water mixtures containing salt forms of 2,4-D by five or more times, but they had little effect on a water mixture of the ester form of 2,4-D.

Staniforth and Loomis believe that a surface-active agent like lauryl sulphate merely accelerates the initial responses to 2,4-D, such as epinasty and stem curvature, and that in the end these detergents do not really increase the herbicidal properties of 2,4-D. This effect may be due to more rapid absorption and translocation of 2,4-D when surface active agents are used than when the growth regulator is applied alone.

Crafts (11) has proposed that herbicides might be divided into 2 general classes, those that are polar and those that are nonpolar. This is of interest here because the growth regulator 2,4-D is now an important herbicide. Crafts concludes on the basis of general results with a variety of herbicides, that polar compounds are most effective when applied to the roots of plants and that nonpolar ones are most effective when applied to the above-ground parts of plants. He suggests that this difference may be due to a difference in the rate of absorption. Since radioactive polar and nonpolar forms of the 2,4-dichlorophenoxy com-

pounds are now available, a means of testing this proposition is at hand.

Regarding the absorption of growth regulators, we can conclude that the uptake of these substances by plants is greatly influenced by such factors as the age of the tissues to which the chemical is applied, light, temperature, and the presence of surface-active substances which tend to increase the rate and extend the period of absorption.

TRANSLOCATION

Two methods have been used to measure translocation of synthetic growth-regulating substances in intact plants: first, evidence of a growth response some distance from the treated area has been used as an indication that the growth regulator was translocated within the plant; second, some growth-regulating substances have been tagged with radioactive isotopes and the course of their movement through the plant followed by means of usual tracer techniques. The path of translocation of growth regulators depends to some extent upon the way the chemical is absorbed by the plant. Taken in through the roots the growth regulator in most instances is moved rapidly upward through the water-conducting tissues (22,35). Absorbed by leaves, however, the chemical is translocated mainly in living cells of the phloem. If, for instance, a few micrograms of 2,4-D are placed on a leaf, the plant can, under some conditions, rapidly absorb and translocate the chemical to the stem where it is moved in both an upward and a downward direction (12).

The amount of growth-regulating substance translocated from a leaf depends upon several factors. Young rapidly growing leaves may absorb 2,4-D but fail to translocate it to other parts of the plant (35). Phenoxy compounds are translocated more readily from leaves of medium age than from either younger or older ones (22,39). Similarly, in stem tissues most marked over-all responses have been observed when the chemical was applied to the young, succulent portion of the stem, least when the chemical was applied to the older, lignified portion near the soil level (24). Movement of 2,4-D from leaves apparently involves the same mechanism as that used by the plant to translocate the products of photosynthesis or a similar one. Growth regulators of the phenoxy type are not translocated from leaves under conditions unfavorable for the production and translocation of photosynthate (25,35,48,58). Thus 2,4-D was not translocated from leaves kept in CO_2-free air or in darkness.

Rice (48) has reported, however, that absorption of 2,4-D by leaves of bean plants was not affected by increasing the light intensity from 100 to 900 foot-candles.

The chemical nature of the growth regulator may in itself influence the rate of its absorption and translocation. The morpholine salt, the butyl ester, and the acid forms of a phenoxy compound (parent compound 2,4-dichloro-5-iodophenoxyacetic acid) have been applied in equal molecular amounts to bean leaves and their absorption and translocation followed by means of radioactive tracers (39). Of the three forms, the acid was translocated in the greatest amount, the salt in the least amount.

Under constant conditions bean leaves apparently absorb and translocate 2,4-D at a relatively uniform rate for a period immediately following treatment. In recent experiments (39) 2,4-D acid was applied in an aqueous mixture containing Tween 20 to bean leaves, 10 micrograms per leaf. On the basis of tracer measurements the amount absorbed and translocated to the stems was essentially linear with time during a period of four days following treatment.

Not all growth-regulating substances are translocated from leaves even under the most favorable conditions. Certain nicotinium compounds, such as 2,4-dichlorobenzylnicotinium chloride, have a systemic regulating effect when applied to succulent stem tissues of bean plants (44). These same compounds were not effective when applied in amounts of even a milligram or more to leaves or to the cotyledons of the plants. There is reason to believe that the method by which plants absorb and translocate different growth-regulating substances may vary widely in some respects, but so far these differences are not understood.

Summarizing the data so far, it is evident that the rate of translocation of growth-regulating substances from a leaf is not related to the rate at which the chemical may be absorbed by the leaf. Translocation of such regulators as the phenoxy compounds is associated in some way with the translocation system involved in the movement of the products of photosynthesis. If activated diffusion plays a part in the translocation of growth regulators, as has been suggested by Clark (10), then such a phenomenon must be governed by external factors, including light and carbon dioxide supply; for without adequate illumination and carbon dioxide, growth-regulating substances are apparently not translocated from leaves of plants.

TISSUE COMPOSITION

After growth-regulating substances have been absorbed and moved to the different parts of the plant they incite specific physiological responses, and these are sharply reflected in or evidenced by the amount and kind of chemical constituents in the plants.

Tissues of stems that respond most easily to such substances as 2,4-D are those that possess a high level of oxidation-reduction activity (18). In bean plants these tissues are phloem, endodermis, cambium, and xylem parenchyma. When 2,4-D, indoleacetic acid, or naphthaleneacetic acid come in contact with these tissues a series of chemical changes is generally set in motion. The end result of these responses depends in part upon the amount and the kind of growth-regulating substances used. If for example, a minute amount of 2,4-D is applied to a sensitive plant, then the chain of responses does not extend far and only those reactions involved in cell elongation may be affected. If more of the compound is applied the chain of responses may be carried on through the process of cell division, the organization of these new cells into tissues, and finally their orientation into organs such as roots.

If we consider this series of responses from the chemical standpoint, the first obvious effect is an increase in the water content of the cell (17,32,7,45), which is paralleled by an increase in the size of the cell. Brown (7) in testing bean plants found that after several days of treatment the water content of leaf tissues was depressed by 2,4-D while that of stem tissues was increased. Thus, the response by some leaf tissues may differ from those of stem tissues with respect to the effect of 2,4-D on their water relations.

Two theories have been proposed to explain why stem tissues take up water when treated with certain types of growth regulators. First, the chemical may bring about the degradation of certain cell constituents so that the osmotic pressure of the sap is increased (17). There is also evidence that cells under the influence of indoleacetic acid, for example, absorbtions, such as potassium, more readily than do untreated cells. Both the absorption of ions and the degradation of cellular constituents would tend to increase osmotic pressure and favor water uptake.

The second theory deals with the effect of growth regulators on the cell wall. The resistance of the wall to extension is thought to be lowered by the chemical so that the cell enlarges, thus allowing absorption of water until a new equilibrium is reached (45).

Argument has been leveled against the theory based on a rapid increase in osmotic pressure. So far it has not been possible to demonstrate experimentally such an increase in osmotic pressure following application of a growth regulator. On the other hand, the theory based on wall extensibility may not entirely explain the phenomenon since it has been repeatedly demonstrated during the last three years that once a cell has expanded and taken up water under the influence of the regulating chemical, the cell has then changed in a way that makes it better able to retain the water it holds against forces of evaporation, than is the case with an untreated cell (32). Wall extensibility would hardly account for this increased water-retaining capacity of treated tissues. Increased osmotic pressure, on the other hand, might in part account for this effect.

There is an abundance of evidence indicating that growth regulators accelerate either directly or indirectly the activity of some enzymes in plants. However, direct proof of their effect on enzyme systems is meager. Berger and Avery (3,4,5) obtained more highly active dehydrogenase from oat plants treated with indoleacetic acid than from untreated ones. Eyster (14,15,16) believes that growth stimulation results from the release of diastase from an inactive to an active form. Gall (18) demonstrated that starch in agar media was more readily digested by enzymes that diffused from sections of bean plants treated with 2,4-D than by enzymes from comparable untreated sections. His results are inconclusive for he states that the growth regulator may either have increased production of enzymes or increased their activity, or the chemical may have affected the tissues so that, although they contained the same amount of enzyme, more of it diffused from the treated than from the untreated section. His work shows clearly, however, that enzyme digestion of starch outside of the treated stems was greater than it was outside the untreated ones.

Within the plant the activity of the enzyme system involved in the conversion of starch to sugar is also affected by some growth-regulating chemicals. In leaves of bean and morning-glory plants, for instance, starch hydrolysis has been accelerated by the application of relatively large amounts of such substances as indoleacetic acid, indolebutyric acid, naphthaleneacetic acid, naphthoxyacetic acid, and 2,4-D (34,38,43). In treated leaves a marked increase in sugar content at first paralleled starch degradation, but as the starch was depleted their sugar content

fell below that of untreated ones (38,50,51). The net result was a depletion of the readily available carbohydrates in leaf tissues. Phenylacetic acid did not accelerate starch degradation in leaves nor did naphthaleneacetamide. The effect of such growth-regulating substances as indoleacetic acid and 2,4-D on reserve carbohydrates in other parts of plants is similar to that described for leaves (1,2,6,34,46,50,56).

Bausor (2) obtained evidence that tomato cuttings retained their starch reserves when kept in darkness and supplied with sugar. Treated plants, on the other hand, utilized their starch reserves irrespective of the external carbohydrate supply. In these same experiments 0.02 per cent indoleacetic acid inhibited starch digestion in thin stem sections but hastened starch digestion in intact stems. This behavior may be explained, however, on the basis of the magnitude of treatment, there being much more growth regulator applied per cell in the case of the sections than in the case of the entire stems.

Hydrolysis of complex carbohydrates, such as hemicelluloses, may also be accelerated by the application of 2,4-D to plants (9). Thus, the action of some hydrolytic enzymes in plants is either directly or indirectly accelerated by some kinds of growth regulators.

Hagen *et al.* (19), on the other hand, have recently shown that the activity of castor bean lipase in hydrolyzing olive oil was less in the presence of small amounts of 2,4-D acid than when used alone. They believe that only the acid form of 2,4-D was directly effective in reducing the lipase activity under their test conditions. Some hydrolytic enzymes, therefore, may under some circumstances be inhibited by the presence of a chemical such as 2,4-D.

Nitrogenous compounds in plants are also affected by the application of growth substances. In general most growth-modifying chemicals bring about an increase in protein and amino acid content of stems when the regulator is applied in relatively large amounts (31,41,42,49). In succulent plants regulating chemicals generally cause a mobilization of nitrogenous constituents towards the basal parts of the stems, both in cuttings and intact plants (2,42,52,55). This is not true, however, of all regulating chemicals. Applied to bean plants, for instance, naphthaleneacetic acid brought about a twelvefold increase in water-soluble nitrogenous compounds in the stems. Naphthaleneacetamide, on the other hand, brought about a decrease in amount of these compounds in the stems (31). Similar differences were also observed in carbohydrate fractions when

the effects of the two compounds were compared. Naphthaleneacetic acid increased starch hydrolysis and the accumulation of sugars. Naphthaleneacetamide caused the simple forms of carbohydrates to be built into more complex forms, such as lignin and cellulose, as was indicated by microscopic examinations of stem sections (31). Thus two regulators that are closely related chemically brought apparently opposing responses when applied to stem tissues.

The mineral content of the stem tissues of bean plants has been increased through the application of growth regulators to the stems (8) and to the roots (8,20). The movement of potassium, magnesium, manganese, and boron into treated regions of stems was increased a measurable amount within 30 hours after indoleacetic acid was applied. Phosphorus and copper were mobilized in treated stem tissues within 48 hours after treatment. Iron and aluminum were also mobilized by treatment but more slowly than were other elements. The calcium content of stems was not affected by treatment with indoleacetic acid.

In these experiments a sufficient amount of the regulator was used to bring about cell proliferation. It is obvious that certain elements were used for the structure of these new cells, and some elements, such as phosphorus, were found to increase in the treated stems at a rate about equal to the increase in total solid substance. On the other hand, there is evidence that some metals, such as copper, magnesium, and iron, are essential to enzyme systems, and it is suggested that these elements accumulate in tissues where meristematic activity has been induced, and play a part in the relatively large amount of enzyme activity involved (8). It is of interest that calcium, which is not abundant in meristematic tissues, was not mobilized as the result of treatment of stems with indoleacetic acid.

Recently Rhodes and Templeton (47) reported that 2-methyl-4-chlorophenoxyacetic acid interfered with the potassium metabolism of rape but not of oats. They suggested that this adverse effect on potassium metabolism may account for the herbicidal effects of 2,4-D and for the difference in sensitivity of these 2 kinds of plants to this type of herbicide.

The effect of 2,4-D and other growth regulators on the composition of fruits has recently received attention. In general, growth regulators tend to hasten starch hydrolysis in the tissues of relatively mature detached fruits like the banana, pear, and apple. With respect to starch

this response is similar to that which occurs in stems of plants treated with these substances (21,40,53).

It was recently found that the accelerating effect of 2,4-D on the ripening of bananas is greatly reduced by the lack of adequate aeration following treatment (29). Although the reason for this is not understood, the results indicate that in the absence or the presence of certain gases, tissues of fruits such as the banana may be relatively insensitive to 2,4-D.

Recently attention has been directed toward the effect of growth-regulating substances on the vitamin content of plants. In recent experiments with beans the initial vitamin C content of the pods was not affected when the plants were sprayed with 4-chlorophenoxyacetic acid. The rate at which the vitamin was broken down during storage of the treated fruits was, however, much slower than for untreated ones. This effect was thought to be indirect since the growth regulator increased the storage life of the pods (36).

According to recent tests with bean plants, application of 2,4-D brought about reduction of thiamin, riboflavin, and nicotinic acid in the leaf tissues (26). Stems of treated plants, on the other hand, contained higher concentrations of thiamin, riboflavin, nicotinic acid, and pantothenic acid than did comparable parts of untreated ones. The concentration of carotene in both leaves and stems was depressed by 2,4-D, while the concentration of pantothenic acid was increased in stem and leaf tissues by its application. These data indicate clearly that 2,4-D brought about definite shifts in the concentrations of certain vitamins between stem and leaf tissues, but information on its effect on total vitamin production per plant is not at present available.

2,4-D has increased the protein content of wheat seedlings when applied in amounts ordinarily used for weed control (13). Increases of approximately 5 per cent or less in the protein content of seeds have been reported on the basis of field tests. Smaller increases were readily observed in greenhouse experiments, but in most instances these were accompanied by a depression in the yield of wheat seeds (33). The effect of growth-regulating substances on the chemical composition of fruits and seeds offers a relatively new and fertile field of study, which is just now beginning to receive the attention it deserves.

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Histological Responses to

Growth-Regulating Substances

J. M. BEAL

Both gross and histological responses of plants to different growth-regulating substances show a rather wide variety of patterns. Not only may plants of different species, genera, and families behave differently, but the age and succulence of individual plants within a variety at the time of treatment and the environmental conditions under which they are grown play important roles in the responses which they make. It is the purpose of the present paper to review the various types of responses to a number of these substances that have been reported in recent years.

Early attempts to analyze the responses of plant tissues to specific chemical substances were made by Erwin F. Smith as far back as 1917 (34). He performed certain experiments in an effort to determine the mechanism of tumor formation resulting from infection with *Phytomonas tumefaciens*, using simple inorganic compounds, mainly ammonium salts dissolved in water. These were injected into the hollow pith of *Ricinus* and similar stems where they induced cellular proliferations of a callus-like character, with the differentiation of some vascular elements.

Interest in plant hormones developed in the late 1920's and information on the gross morphological responses of plants to such substances accumulated rapidly. Boysen Jensen, Avery, and Burkholder (8) have presented the observations recorded up to 1936. Zimmerman and Wilcoxon (40), Cooper (12), and Brown and Gardner (9) had reported the production of tumors following the application of indoleacetic acid to plants. Laibach and Fischnich (25) reported the effects of an indole-acetic acid-lanolin mixture on the stems of *Coleus*, *Vicia faba*, and tomato.

In the treated stems of *Coleus* Fischnich (14) found that the cells of the parenchyma enlarged, the cells in the vicinity of the cambium proliferated, the cambium became active, new vascular bundles appeared between the old ones, and adventitious roots arose principally in the borders of the vascular bundles. Czaja (13) studied the effects of indoleacetic acid-lanolin paste on *Helianthus* hypocotyls and described some of the tissue responses. Snow (35) showed that auxin-a, indoleacetic acid, and extracts of urine stimulated cambial activity in *Helianthus* seedlings.

Relatively little detailed description of the histological responses of plants to any growth-regulating substance had appeared, however, prior to the publication of the paper by Kraus, Brown, and Hamner in 1936 (23), dealing with the gross and histological reactions of the red kidney bean to applications of indoleacetic acid. This paper stimulated tremendous interest and gave direction for numerous papers which followed, dealing not only with the responses induced by indoleacetic acid but with a number of other chemical substances. Because of the accurate and detailed observations presented, as well as of the influence exerted on subsequent histological studies following the application of indoleacetic acid and other growth-regulating substances, it is deemed advisable to point out some of the most significant of their findings.

RESPONSES OF YOUNG DECAPITATED STEMS TO INDOLEACETIC ACID

The indoleacetic acid was applied as a 3 per cent mixture in lanolin to the cut surface of the second internode of young bean plants which had been cut off squarely about 1 mm. below the base of the petiole of the first compound leaf. In some instances axillary shoots were used. Essentially the same responses were obtained in both cases.

Following decapitation and application of the lanolin mixture, little gross response of the stem was noticeable within 18 hours. Swelling of the stem for 1 to 2 mm. down from the cut surface then began, and by the end of 48 hours the topmost portion of the stem had become distinctly enlarged. At 72 hours the end of the stem attained a diameter nearly twice that at 5 mm. below the cut surface, and by the end of 110 to 120 hours glistening tips of root primordia were visible about the periphery of the tumor. At 144 to 168 hours the roots were more evident and some of them emerged from the surface of the tumor. Such tumors may continue to grow for periods longer than six months and frequently attained

diameters of 2 cm. or more, and a height above the original cut surfaces of 2–2.5 cm. Similar appearing tumors were induced on bean pods, with roots developing from them also.

Histological Details.—The cells of the epidermis and pericycle responded less actively than those of the other tissues. The cells of the cortical parenchyma enlarged somewhat and those near the endodermis became meristematic. The cells of the endodermis were highly responsive; they were the first to show meristematic activity with nuclear divisions being greatly accelerated shortly after treatment, and extending as far as 5 cm. below the point of application after a week. The derivatives differentiated as phloem and xylem elements and large, apparently multinucleate, parenchymatous cells. Many derivatives remained meristematic while others gave rise to root histogens and eventually to adventitious roots. Especially over the vascular bundles long proliferating strands of vascular tissues developed from endodermal derivatives. These frequently enlarged sufficiently to rupture the tissues exterior to them. The parenchyma of the primary phloem showed the same general type of response and subsequent differentiation of tissues. Some of the cells derived from it formed a part of the cortical tissues of the apical crown of adventitious roots. A part of the tissues composing the cortical portion of these adventitious roots, however, was derived from the parenchyma of the secondary phloem. Other cells matured into parenchymatous tissue, tracheids with simple pits, sieve tubes, and companion cells. The cambium divided actively. Its derivatives often remained active over long periods of time, later maturing as various phloem or xylem elements, or continued as meristematic zones from which such elements continued to form.

Near the surface of application the cells of the rays adjacent to the xylem proliferated greatly. Many of their derivatives matured as tracheids, with meristematic areas intermingled with them. This confused mass of tissue often persisted and continued development for weeks. The ray cells adjacent to the phloem and just within the pericycle also proliferated greatly and, in conjunction with the phloem cells flanking them, gave rise to root histogens and also directly formed large portions of the adventitious roots.

The pith cells also proliferated greatly, with the first marked activity adjacent to the surface of application, but later activity progressed down the stem next to the elements of the primary xylem. Large pitted

tracheids intermingled with large parenchymatous cells and meristematic zones continued to develop for a long period of time and accounted for much of the overgrowths which continue development for periods longer than six months (20).

Decapitated stems treated only with lanolin generally enlarged but little. When decapitated and otherwise untreated, calluses derived mainly from cells of the primary and secondary phloem parenchyma might be formed.

The main responses shown by various tissues of the tomato (7,27), cabbage (17), pea (33), and broad bean (32) to indoleacetic acid were similar to those in the bean, differing from it and from one another chiefly in degree and a few details.

Decapitated and treated stems of etiolated peas (32) developed a meristem cylinder from division of the cells of the inner cortex, endodermis, pericycle, and phloem parenchyma. The root primordia varied in origin in younger and older stems. In younger seedlings the identity of the endodermis became lost in the meristem cylinder complex. The root primordia arose from a group of ray cells within this complex. In older stems the endodermal cells did not divide and the root primordia were pericyclic and intrapericyclic in origin. *Iresine* (21) and *Mirabilis* (19) differed from most other species investigated in that the pericycle was more responsive than the endodermis. The phloem of *Iresine* was strongly reactive while that of *Mirabilis* was not. Adventitious roots developed mostly from pericycle and ray tissues.

In *Vicia faba* (32) adventitious roots were rarely formed, although large, rather unorganized masses of meristematic tissue occasionally developed. These appeared to be potential roots, which had not organized meristems or orderly systems of derivatives. The cabbage plant (17) was interesting because it developed adventitious shoots from the tumors; apical ones from callus, and lateral ones from inner cortex and ray tissues.

All of the preceding plants are dicotyledons. The only detailed histological investigation of the responses of monocotyledonous stems to indoleacetic acid was that on three species of *Lilium* (3). In *L. philippinense formosanum* and *L. longiflorum* the first detectable changes in decapitated stems occurred in the cells of the fundamental parenchyma, lateral and centifugal to the vascular bundles, usually only the outer bundles. These cells became meristematic, and by repeated divisions gave rise to cells which later differentiated as adventitious roots. In *L. harrisi* (*L. longi-*

florum var. *eximium*) the region of response was limited largely to the cells of the epidermis and outer cortex in the immediate vicinity of the leaf axils adjacent to the surface of application. Adventitious roots were rarely formed. The epidermal cells of the stem immediately above the axil elongated radially, while the outer cortical cells centripetal to as well as slightly below them enlarged and in the course of about five days began dividing. This marked the initiation of the region at which buds later developed. Neither buds nor bud primordia were present in these leaf axils at the time of treatment. The groups of meristematic cells in the leaf axils divided repeatedly, accompanied by radial divisions of the cells of the epidermis over them. The subsequent growth of their derivatives resulted in a hump of cells which through further development and differentiation became a bud. As many as three buds may develop in one axil. If permitted to grow on the plant for 8 to 10 weeks, the induced buds became bulbils with roots at their bases. Bulbils were removed from the parent plant and grown separately. No visible anatomical differences were observed which could be used in explaining the differences in the behavior of the three species.

Responses to Other Growth Substances

A number of other growth substances have been applied to the red kidney bean. Indolebutyric acid (20) produced apical tumors similar to those resulting from indoleacetic acid, but with fewer, thicker roots in the upper zone, more roots 1 cm. or so below the treated surface, and several tiers of roots between these two regions. Naphthaleneacetic acid (20) resulted in the formation of apical tumors with greater and more uniform diameter for a distance of about 1 cm. below the cut surface, where a circle of large root primordia was formed, each primordium over one of the main vascular bundles. The responses to tryptophan (22) are less vigorous but similar insofar as the responsive tissues are concerned. No adventitious roots developed but abundant anastomosing vascular strands were differentiated, mostly from derivatives of the endodermis. Whether these effects are due to indoleacetic acid derived from tryptophan is not wholly certain. Histological studies indicate they may not be; biochemical determination of material *in situ* must be made. An extract of maize pollen (28) applied to bean stems produced responses similar to those from tryptophan. Tetrahydrofurfurylbutyrate (29) produced little or no effect on epidermis, cortex, pericycle, or cambium,

and only a slight effect on the endodermis. Xylem, phloem, and rays proliferated, but no roots developed. Naphthoxyacetic acid (2) applied to tomato stems produced responses similar to those from indoleacetic acid. Root primordia resulted from the activity of the pericycle and phloem parenchyma, the root cap arising from the endodermis.

The next important group of compounds to be investigated was the substituted phenoxy group. These compounds attracted great interest because of their marked formative and telomorphic effects, as well as the fact that many plants treated with them are killed. Numerous investigations on the reactions of plants to them led to their use as selective herbicides. Recently some of them have been employed for several other important purposes.

The first report dealing with the histological responses induced by any of these substances was a rather brief description of the histological changes in bindweed and sow thistle following applications of 2,4-dichlorophenoxyacetic acid (2,4-D) in herbicidal concentrations (37). Following this there were three papers (5,6,36) dealing with the reactions of the bean to these compounds, especially 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). In two of these reports (5,6) both lanolin and Carbowax 1500 had been used as carriers of the growth substances, and it was found that the carrier played a role in the effectiveness of some of the substances. In general the tissue responses were similar in kind but greater in degree than those induced by indoleacetic acid and several of the other substances. Endodermis, cambium, phloem, and ray parenchyma were strongly activated, and in most instances roots developed abundantly, mainly from the phloem and ray parenchyma.

Somewhat similar experiments were done on decapitated bean plants, using 2,4-D and four of its salts, namely, ammonium, copper, calcium, and magnesium 2,4-dichlorophenoxyacetates (30). Histological responses in the early stages showed considerable similarity. Later certain differences became evident, but no response induced by one substance was entirely absent in the reaction to another substance, since all responses appeared to fall within the range of effects distinctive for 2,4-D.

A 2 per cent phenylacetic acid and lanolin mixture (38) applied to the cut surface of decapitated young bean plants produced flat-topped, somewhat tuberculate tumors. The bulk of the tumor arose from a marked proliferation of the inner cortex, endodermis, and primary phloem parenchyma. The derivatives matured as tracheids or paren-

chyma, continued as patches of meristematic tissue, or infrequently differentiated as small vascular bundles. There was increased activity of the cambium, its derivatives maturing entirely as tracheids. Root primordia were rare.

Alpha-naphthylmethylacetate applied to the cut surfaces of decapitated second internodes of the bean (1) produced in general the same tissue responses as had occurred following the application of most other growth-regulating substances. The production of considerable wood was one of the notable responses. In this respect it resembled the response to α-naphthylacetamide (24).

Responses of Roots

Studies of the effects of growth-regulating substances on roots were for a time limited largely to observations on the stimulation or inhibition of root elongation, the development of laterals, and the accompanying physiological activities. Some histological and cytological responses of *Allium* roots to several chemicals were described by Levan (26). Noirfalise (31) reported the cessation of elongation of the primary root of young seedlings of *Vicia faba* when placed in certain aqueous solutions of heteroauxin. Relatively high concentrations inhibited elongation but resulted in marked increase in diameter and the production of numerous lateral roots. Microscopic preparations showed that the increase in diameter resulted chiefly from the activity of the cells of the pericycle. Morphological changes in induced roots of wheat following treatment with heteroauxin were reported by Burström (10). A study of the histological and cytological changes induced in the roots of *Allium cepa*, *Narcissus* (var. Paper White), and *Tulipa* (vars. John Ruskin and Louis XIV) by several growth-regulating chemicals was presented by Carlton (11). Six substances were used: α-naphthaleneacetic acid, indoleacetic acid, indolebutyric acid, β-naphthoxyacetic acid, α-naphthylacetamide, and tryptophan. These were used at concentrations of 10 or 20 parts per million in a three-salt solution. The bulbs were rooted by placing their bases in water, and when the roots had attained a suitable length the bulbs were transferred to the salt solution containing the respective growth substance. Root elongation was inhibited temporarily by indoleacetic acid and permanently by all the others. The roots of *Allium* and *Narcissus* thickened markedly just back of their growing points as a result of treatment with indoleacetic, indolebutyric, naphthaleneacetic,

and naphthoxyacetic acids, and to a lesser extent in the tryptophan. Roots of *Tulipa* showed little enlargement and no adventitious root formation. In *Allium* and *Tulipa* proliferation of the pericycle occurred with the formation of numerous root primordia in *Allium*. Neither true proliferation nor root primordia occurred in *Narcissus*.

RESPONSES OF FRUITS

Parthenocarpy induced by growth-regulating substances was first accomplished by Gustafson (18) in 1936 by applying known chemical compounds in lanolin to the pistils of a number of plants. The following year Gardner and Marth (16) reported the induction of parthenocarpy in American holly, *Ilex opaca*, by spraying the open blossoms with dilute aqueous solutions of several growth substances, including indoleacetic, indolepropionic, indolebutyric, and naphthaleneacetic acids. Gardner and Kraus (15) investigated the histological changes of the pistil of the holly as affected by indoleacetic acid and found that the development of the parthenocarpic fruits paralleled almost precisely that following pollination. The chief differences were that the stigmas of the sprayed fruits proliferated somewhat more than those pollinated, did not collapse and suberize as soon, and developed neither embryo nor endosperm in the ovules.

Beal (4) applied 1 per cent growth substance and lanolin mixtures shortly after anthesis to the cut surface of ovaries of *Lilium regale* from which the stigmas and styles had been removed by cutting squarely across the top of the ovary at the base of the style. Indoleacetic, naphthaleneacetic, and naphthoxyacetic acids and a combination consisting of equal volumes of the three 1 per cent concentrations thoroughly mixed were used.

Ovaries to which the three, as well as those to which the combination were applied, enlarged in length and diameter at approximately the same rate and attained nearly the same final size. Their length was as great as that of fruits resulting from pollination, but their diameter was always less. Neither apical tumors nor adventitious roots were formed.

A transection of an ovary at 795 hours (33 days) after treatment showed well-developed carpel walls and ovules. The greater part of the growth had resulted from enlargement of cells, although a few cell divisions had occurred in some regions of both the carpels and ovules following treatment. Again neither embryos nor endosperm were formed.

A naturally parthenocarpic variety of cucumber similarly cut at the base of the style and smeared with 2 per cent indoleacetic acid and lanolin mixture behaved rather differently according to Young (39). If treated at full bloom the tissues adjacent to the surface of application remained alive but showed slight proliferation. Ovules were intermediate in size between those of controls and those given the prebloom treatment with indoleacetic acid. If treated about 4 days before full bloom, tissues of the nectary, floral tube, and neck proliferated to form a small apical tumor in which no vascular bundles or root primordia appear. Ovules developed to about half normal size, and seed coats became partially hardened.

Application of a 3 per cent indoleacetic acid and lanolin mixture to the cut surfaces of partially mature bean pods which had had their tips removed resulted in the production of large vascular apical tumors and roots (20). The tissues composing these tumors were derived mainly from the proliferated exocarp and mesocarp of the pod. The endocarp, which in untreated pods is principally derived from proliferated epidermal cells, became somewhat more active in treated pods, but formed little or no part of the apical tumor. Vascular elements apparently were not differentiated from this tissue. The large apical tumors are built up from derivatives of cells near the treated surface, a far shorter distance than was the case in stems. The seeds aborted in at least half the apically treated pods; the larger the tumor the fewer the seeds. This appeared to result from the mobilized materials that entered the pod being diverted to the tumor rather than to the seeds.

Lateral Application to Stems

Hamner and Kraus (20) made lateral applications of a 3 per cent indoleacetic acid and lanolin mixture to bean stems by drawing the mixture out as a thin thread which was then laid on the second internode to encircle it completely. Care was taken to avoid injury to the stems.

Marked yellowing and swelling occurred 2 or 3 mm. above, below, and at the line of application. The effects increased with time until by the end of a week or ten days the tumors were often 2 cm. or more long, spindle shaped, and markedly ridged over the vascular bundles. Roots in longitudinal rows emerged mainly between the swellings over the vascular bundles.

The histological responses closely resembled those of stems decapitated

and treated terminally. The pith showed practically no meristematic response, but the cells of the inner cortex, endodermis, primary phloem, cambium, and the rays flanking the phloem showed marked activity. The roots which developed were related to the rays in the same manner as those developed in apical tumors of decapitated plants.

These responses indicate that almost any parenchymatous tissues may become meristematic when growth-regulating substances are applied to them. It is obvious also that the same general types of responses are incited by a large number of these substances, although there are marked differences in the degree or intensity of responses of the several tissues in relation to the specific substances. The difference in response with respect to the kind of substance employed is greater than it is to the concentration of a single substance applied over a rather wide range of the higher concentrations. It is, however, impossible to say just what the concentration of the substance which reaches any cell or group of cells may be when applications are made in the various ways listed. There is no doubt that the substances are soluble and that they diffuse into and among the cells of the plant, but the rate at which this may occur is difficult to determine.

Thus it would appear that:

1. In the presence of growth substances and proper food and nutrient supply, cells of recognizably differentiated tissue systems may dedifferentiate, become embryonic, and proliferate. From these derivatives, tissues quite distinct in type may be differentiated (for example, vascular bundles from phloem, endodermis, pith, etc.).

2. In general not wholly new responses are evoked, because many of the tissue systems respond similarly to changes in nutrients and food supply, and to wounding and other environmental changes (for example, healing of wounds, rooting of cuttings, delay of abscission).

3. Although no genuine new tissues arise the nature of their distribution and proportions and pattern may be profoundly affected.

4. To this extent morphogenesis is brought under control, or may be manipulated, as is now done in so many instances in the agricultural field by the use of these compounds.

5. The course of development apparently is changed, but more than a growth substance is required; foods and nutrients play important roles in the quantity and quality of response.

6. Age of tissues, or perhaps more basically the chemical nature of

the tissues correlated with aging, plays a major role in the type and quantity of response.

7. It is as important to know the qualities of the tissues that are to respond as it is to know the growth substance to be employed, since to many of the substances the responses are very similar while to others they may be quite different, as for example to naphthaleneacetic acid and naphthylacetamide. Thus the growth-regulating substance can be regarded only as a releaser (or in part a controlling means) of response of the living cell, which still remains the critical entity, and not as the prime inciter itself.

8. While in morphogenetic studies genetic capacity may control, yet growth-regulating substances determine the degree to which expression does or may take place. They are one of a complex group of factors, including light, temperature, food and nutrient supply, and other environmental conditions. Therefore growth regulator or Ergocrine is a more precise use of terminology than growth substance or even hormone, because many of the effects of these substances suppress certain physiological reactions as well as excite them. The same is true for the morphological responses and final visible changes as well.

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Comparative Effects of Growth Substances on

Stem Anatomy

B. ESTHER STRUCKMEYER

M ANY different growth substances have been tested in attempts to determine the nature of their effect on plant growth (10). It has been found that growth substances may have characteristically different effects on a plant, particularly on the anatomical structure of the stem. Thus, in studies carried out here, 2,4-dichlorophenoxyacetic acid (2,4-D) stimulated cell proliferation and lateral root formation (9); α-naphthaleneacetamide induced cambial activity resulting in the formation of more xylem and in a thickening of the cell walls of the xylem and the phloem fibers (4); and β-naphthoxyacetic acid was particularly effective in producing parthenocarpic tomato fruits (7). Alpha-naphthaleneacetic acid has been reported to prevent the preharvest drop of apples in the fall as well as to thin apples shortly after blossoming in the spring (3,8).

In our studies of the anatomical structure of plants treated with growth substances the following problems have been given special attention: 1) why do most dicotyledonous and only some monocotyledonous plants respond to 2,4-D; 2) what is the possible role of α-naphthaleneacetamide in delaying calcium deficiency symptoms; 3) and how does α-naphthaleneacetic acid function in the thinning of apples at one stage and in preventing them from dropping at a mature stage?

Much work has been done on 2,4-D regarding its practical value as a weed-killer. It has recently been reported that some species of dicotyledons and monocotyledons responded to 2,4-D while others did not (2,6). The structure of the stem of young kidney bean plants treated with 2,4-D was reported by Swanson (9). In our investigations older plants were used (six internodes or more) belonging to the monocotyledonous and

dicotyledonous plants. They were sprayed with .01 per cent aqueous solution of 2,4-D.

All the plants were in a vegetative stage when treated. Samples of the stem of monocotyledonous plants were taken in the region that showed a response to the growth substance, such as swelling of the stems or the presence of lateral roots. The dicotyledonous plants were sampled at the second through the eighth internode from the stem tip. Proliferation of cells was apparent in some plants soon after treatment, while in others several days elapsed before a response was evident. The dicotyledonous plants usually made a greater anatomical response to 2,4-D than did the monocotyledonous plants.

Tomato plants were sensitive to this growth regulator. Eighteen days after treatment lateral roots had emerged through the cortex. The cells of the phloem parenchyma, cambium, ray parenchyma, and pericycle proliferated to form root primordia. Proliferation was not limited to the interfasicular region as it was in plants such as cocklebur, for bands of active cells encircled the outer stelar region (Fig. 1, 2).

Fourteen days after treatment with 2,4-D cell division had occurred in some of the cortical cells of *Peperomia;* but the cells most sensitive to this treatment were those adjacent to the vascular bundles, par-

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LEGEND FOR FIGURES 1 TO 13

Figure 1. Fourth internode of tomato control plant. Figure 2. Fourth internode of tomato eighteen days after treatment with 2,4-D; band of lateral roots formed around stem. Figure 3. Fourth internode of control plant of *Peperomia*. Figure 4. Treated stems of *Peperomia* responded to 2,4-D; proliferation of cells and adventitious root formation was apparent. Figure 5. Second internode of cocklebur 97 hours after treatment with 2,4-D showing proliferation of cells in the interfasicular region of the stem. Figure 6. Eighth internode of cocklebur 25 days after treatment; lateral roots emerging through the cortex from the interfasicular regions. Figure 7. Cross section of a leaf 25 days after treatment with 2,4-D; leaf swollen and root primordia differentiating. Figure 8. Stem tip of cocklebur 25 days after treatment with 2,4-D; cells of leaves surrounding stem tip have proliferated, stem tip has broadened, and cells below apex have become necrotic. Figure 9. Stem tip of normal plant. Figure 10. Fourth internode of *Coleus* 35 days after treatment; root primordia formed from fasicular region of stem. Figure 11. Fourth internode of untreated stem of *Coleus*. Figure 12. Untreated plant of *Dracena* showing cambial region. Figure 13. Lateral roots from stem of *Dracena* 24 days after treatment with 2,4-D.

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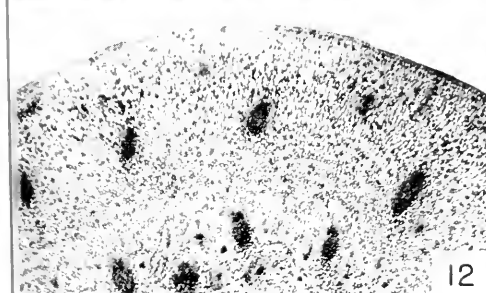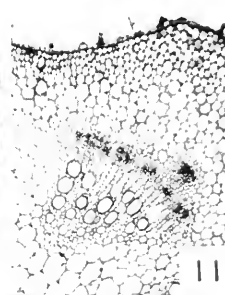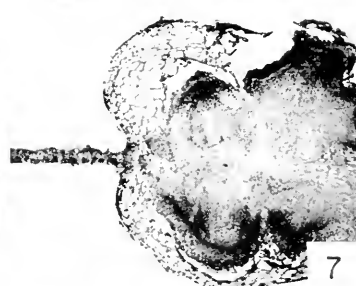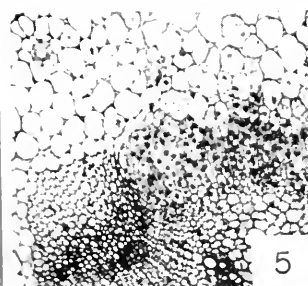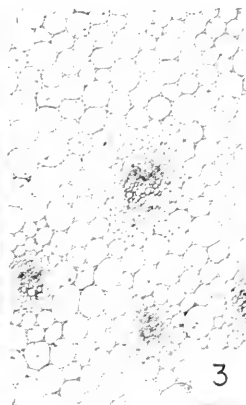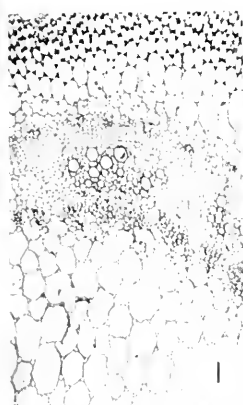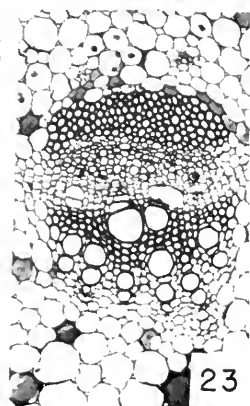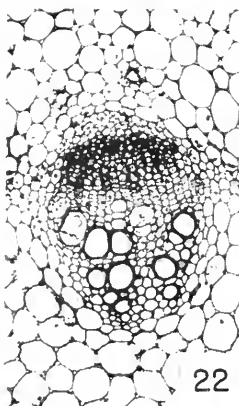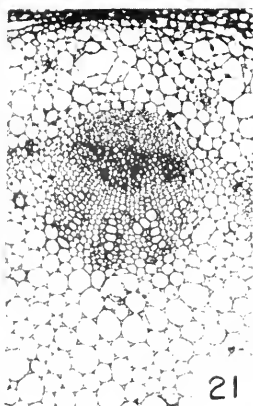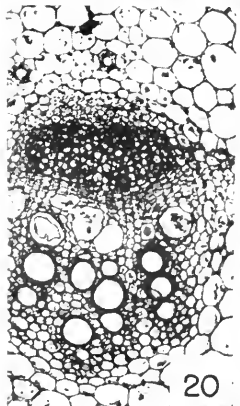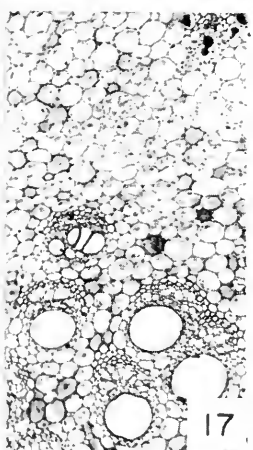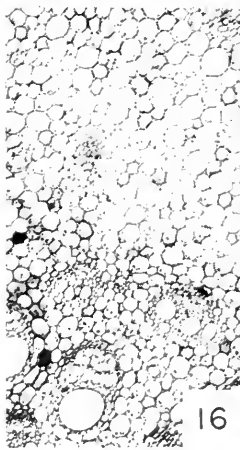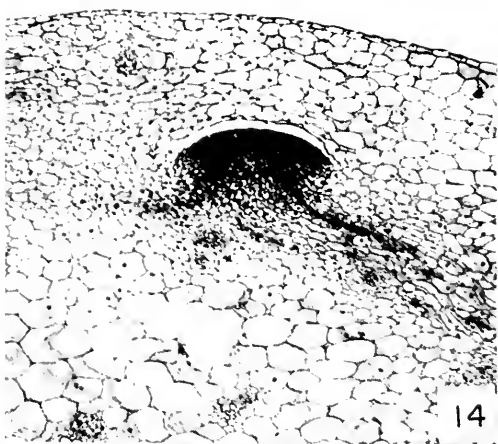
ticularly those next to the phloem which were probably phloem paren-chyma cells and pericycle cells. Thirty-two days after treatment roots developed from the phloem side of the vascular bundle were emerging through the cortex and epidermis (Fig. 3, 4).

Ninety-six hours after treatment proliferation of cells was apparent in the interfasicular region of the second internode from the stem tip of cocklebur. Only a few scattered cell divisions were apparent in the cortex (Fig. 5). Twenty-five days after treatment roots had extended beyond the cortex (Fig. 6). Unlike tomato, where proliferation occurred anywhere in the cambium of the stem, lateral roots were formed only from the interfasicular region of the stem of cocklebur. This resulted in a misplacement of the vascular bundles in the stem perhaps due to the pressure of the developing roots extending through the cortex. The leaves and stem tips of cocklebur were also studied. The stem tip had enlarged to several times its normal size, the apex being broader and the surrounding leaves showing masses of proliferating cells (Fig. 8, 9). Proliferating cells forming root primordia gave rise to the swellings of the veins and midrib of the leaves (Fig. 7).

Coleus responded to 2,4-D in that large root primordia were formed from the fasicular region of the stele. The cambium, phloem parenchyma, and ray cells proliferated to form root primordia (Fig. 10, 11). Poinsettia also gave a marked response to 2,4-D, which appeared later than in the above species. Other species such as sweet potato, *Oxalis*, lambs'-

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LEGEND FOR FIGURES 14 TO 23

Figure 14. Cross section from normal plant of *Tradescantia*. Figure 15. Cross section of stem of *Tradescantia* after treatment with 2,4-D; lateral roots appear to differentiate from the meristematic zone near the periphery of the stem. Figure 16. Cross section of normal stem of *Philodendron*. Figure 17. Cross section of *Philodendron* stem which failed to respond to 2,4-D. Figure 18. Cross section of normal quack-grass rhizome. Figure 19. Rhizome of quack grass treated with 2,4-D. Band of lateral roots encircled rhizome which differentiated from meristematic zone. Figure 20. Fourth internode of cockle-bur grown at a temperature of 70°C. with complete nutrient solution. Figure 21. Fourth internode of cocklebur grown with nutrient lacking calcium. Figure 22. Fourth internode of cocklebur grown in a nutrient lacking calcium but treated with α-naphthaleneacetamide. Figure 23. Fourth internode of cocklebur grown with a complete nutrient supply and treated with α-naph-thaleneacetamide.

quarters. pigweed, and *Verbena* likewise responded to 2,4-D; and the tissues responsible for proliferation and root formation were the same as those for the previous plants mentioned, namely the cambium, phloem parenchyma, ray cells, and pericycle.

Comparing the monocotyledonous plants with the dicotyledonous plants, the same tissues proliferated to form root primordia. *Dracena* developed a swelling at the base of the leaves which perhaps included several nodes and internodes. Upon examination of this region it was apparent that a cambial-like region was present in this plant (Fig. 12). Eighteen days after treatment lateral roots originating in the cambial zone and in the parenchyma cells on either side of it (Fig. 13) were extending through the cortex.

Adventitious roots in stems of *Tradescantia* treated with 2,4-D were formed from the meristematic zone near the periphery of the stem (Fig. 14, 15).

Philodendron failed to respond to treatment with 2,4-D. Vascular bundles were distributed throughout the stem, the larger ones occupying the center. Over the phloem of each bundle was a cap of thick-walled fibers which were perhaps too differentiated to revert to an active stage of proliferation. Although there were parenchyma cells in the stem which ordinarily responded in other species they failed to do so in *Philodendron* (Fig. 16, 17).

Although the above ground stem of quack grass was not affected by this growth substance, the rhizome made a striking response. The parenchyma cells adjacent to the differentiating bundles proliferated and were responsible for the formation of lateral roots (Fig. 18, 19).

The anatomical responses of plants to 2,4-D were similar regardless of whether they were dicotyledonous or monocotyledonous (1,5). Proliferation of cells and root formation from the cambium, parenchyma cells, ray cells, and pericycle were common to both groups.

From these results it has been concluded that whether a plant can be stimulated generally depends on whether there are cells in the stem which are immature enough to proliferate when treated with 2,4-D. These types of cells were more limited in amount in monocotyledons than in dicotyledons, which may explain why the monocotyledons treated with 2,4-D usually recovered.

Another growth substance which had a quite different effect on the anatomical structure than 2,4-D was α-naphthaleneacetamide. Mitchell

(4) reported its effect on cell structure in bean plants. With the application of this growth substance there was increased cambial activity in the stem resulting in the formation of thick-walled xylem cells. A greater number of lignified cells were present as compared with the control.

It was observed that stems of cocklebur grown without a calcium supply generally had smaller and thinner-walled cells. This was especially conspicuous in the xylem cells, xylem parenchyma cells, and pericycle fibers where there was decreased lignification. In fact vessels frequently collapsed because of lack of rigidity of the cell wall. Alpha-naphthalene-acetamide induced a thickening and lignification of cell walls when sprayed on or applied in lanolin to the plants grown without calcium. The first experiment was conducted at a temperature of 70° F. The plants were eight inches tall when the treatment was started. A series of cocklebur plants was set up, some of which were given a complete nutrient solution, a nutrient lacking calcium, a complete nutrient and a spray treatment with α-naphthaleneacetamide, and a nutrient lacking calcium but also receiving an α-naphthaleneacetamide treatment. Thirty-six days from the start of the experiment severe symptoms were apparent in plants grown without calcium. At the same time plants grown without calcium but sprayed with α-naphthaleneacetamide showed no symptoms like curling of the leaves near the stem apex or a spotted appearance of the leaves. Plants given the other two treatments appeared normal.

The anatomical structure of the fourth internode of plants given a complete nutrient supply showed the usual organization of the cells (Fig. 20). An active cambium differentiating phloem and xylem was apparent. Plants lacking calcium in the nutrient solution showed all cells to be smaller and thinner walled; the xylem elements were fewer and less lignified; the vascular bundles were small with few vessels; and the cambium was less conspicuous (Fig. 21).

Plants given a complete nutrient solution and treated with α-naphthaleneacetamide showed an active cambium differentiating vascular elements (Fig. 23). Xylem elements, especially tracheids, were thicker walled than in the control, and increased thickening of the phloem fibers was also evident. Plants grown without calcium and treated with α-naphthaleneacetamide showed no deficiency symptoms thirty-six days after treatment. The fourth internode of these plants showed anatomical characteristics resembling those of normal untreated plants. The vascular bundles were slightly smaller than in the control plants, but the

cambium was just as active. There was a greater lignification of the vascular elements and the fibers, and the cells were much larger than in plants grown without calcium (Fig. 22). Except for the size of the vascular bundles, the structure of the control plants and of plants grown without calcium but treated with α-naphthaleneacetamide was very similar.

During the winter of 1948 the experiment was repeated under long-day photoperiod and at a temperature of 65° F. Twenty days after the start of the experiment deficiency symptoms were apparent in plants grown without calcium. Twenty-four days after treatment deficiency symptoms were also becoming apparent on plants grown without calcium but treated with α-naphthaleneacetamide. After forty-four days plants grown without calcium, and those grown without calcium but sprayed with α-naphthaleneacetamide showed severe calcium deficiency symptoms. The plants were sampled for anatomical observations at this time. The fourth internode showed structural abnormalities typical of calcium deprivation in plants grown without calcium and plants grown without calcium but treated with α-naphthaleneacetamide (Fig. 24, 25, 26, 27). The experiment was repeated and similar results were obtained.

The question then arose as to why results similar to those obtained the previous late spring at 70° F. were not secured. Could the difference in temperature account for the difference in the effect of α-naphthalene-acetamide on delaying calcium deficiency symptoms?

The experiment was again repeated in the greenhouse, except that it was carried out at a temperature of 75° F. After twelve days plants grown without calcium showed symptoms which rapidly became more severe. After sixty days the plants grown without calcium were dead and those grown without calcium but treated with α-naphthalene-acetamide showed no calcium deficiency symptoms. After seventy-five days only slight symptoms of calcium deficiency were apparent in plants grown without calcium but treated with α-naphthaleneacetamide. At this time all plants were sampled for anatomical observations.

The anatomical structure of the fourth internodes of these plants corresponded closely to those of the first experiment at the warmer temperature. The stems of plants grown without calcium had small bundles and small cells, resulting in a decreased amount of lignification. Plants grown without calcium but treated with α-naphthaleneacetamide showed a structure more closely resembling the normal plant.
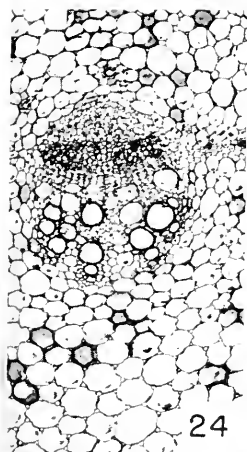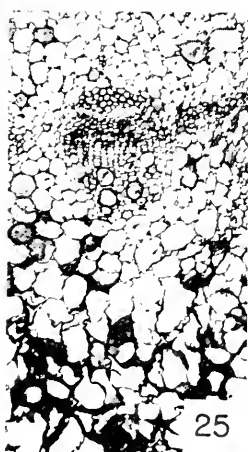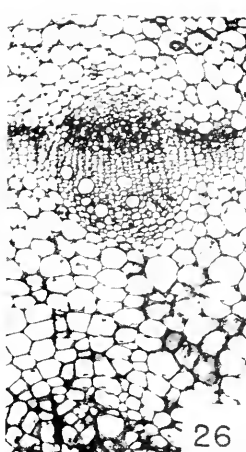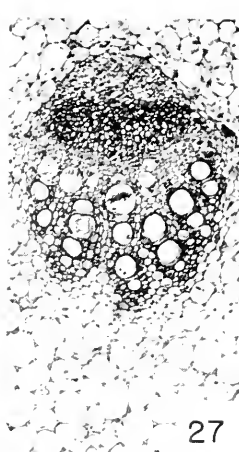
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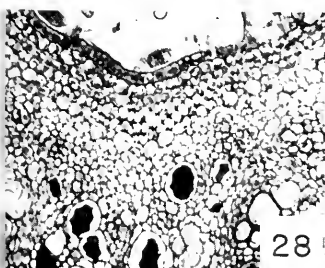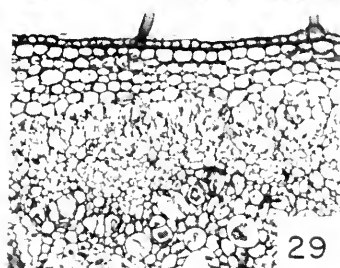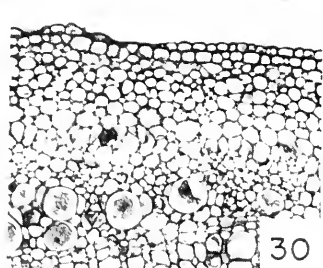
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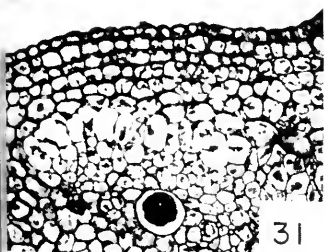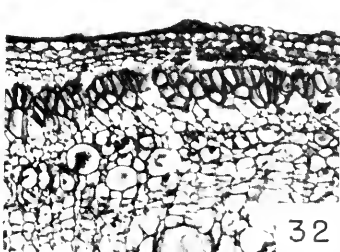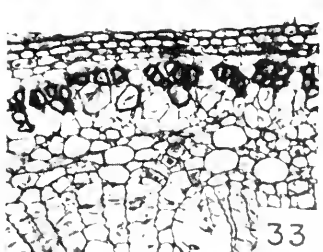
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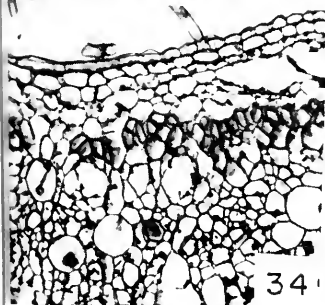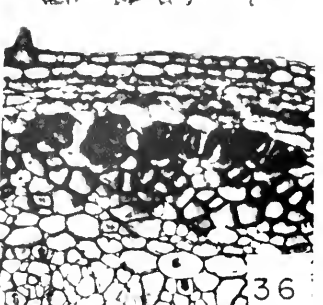
34   35   36

LEGEND FOR FIGURES 24 TO 36

Figures 24–27. Fourth internode of cocklebur grown at a temperature of 65°C. Figure 24. Stem of plant grown with complete nutrient supply. Figure 25. Stem of plant grown without calcium. Figure 26. Stem of plant grown without calcium and treated with α-naphthaleneacetamide. Figure 27. Stem of plant grown with calcium and treated with α-naphthaleneacetamide. Figures 28–30. Second internodes of hemp. Figure 28. Stem of control plant. Figure 29. Stem of plant given an aqueous spray of α-naphthaleneacetamide. Figure 30. Stem of plant treated with α-naphthaleneacetamide in lanolin. Figures 31–33. Fourth internodes of hemp. Figure 31. Stem of control plant; no evidence of thickening of the fibers. Figure 32. Stem of plant given aqueous spray of α-naphthaleneacetamide; fibers have become thick-walled. Figure 33. Stem of plant treated with α-naphthaleneacetamide in lanolin. Figures 34–36. Sixth internodes of hemp. Figure 34. Stem of control plant; fibers have started to put on thick walls. Figure 35. Stem of plant given aqueous spray of α-naphthaleneacetamide; fibers have become thick-walled. Figure 36. Stem of plant treated with α-naphthaleneacetamide in lanolin; shows greater number and greater thickening of fibers.

Sections of the stem were also prepared for microincineration. The mineral residue was recorded by means of photomicrographs to determine the distribution of minerals. The greatest amount of mineral residue was present in stems grown with a complete nutrient solution and treated with α-naphthaleneacetamide; stems of plants grown with a complete nutrient supply had the next greatest amount; stems of plants grown without calcium but treated with α-naphthaleneacetamide had a mineral distribution closely resembling the control plants; and stems of plants grown without calcium showed the least amount of mineral residue. When microchemical tests for calcium were made with 2 per cent sulphuric acid, a positive test was obtained for all four treatments. The exact role of α-naphthaleneacetamide in delaying calcium deficiency symptoms is not known. It may permit greater uptake of calcium from the soil or there may be a conversion of calcium from a relatively immobile to a mobile form. The available supply of calcium would then make possible normal growth of cells.

Since α-naphthaleneacetamide induces cambial activity, a greater thickening of cell walls, and lignification, hemp plants were sprayed with an aqueous solution of α-naphthaleneacetamide and others received an application of this growth substance in lanolin to the fourth internode from the stem tip. The plants were in a vegetative condition when the treatments were made. Sixteen days after treatment the plants were sampled for anatomical studies. The second internodes of the controls and those treated with α-naphthaleneacetamide showed no thickening of the fibers (Fig. 28, 29, 30). The fourth internode of the control plants showed no thickening of the fibers (Fig. 31); whereas the stems treated with α-naphthaleneacetamide showed a thickening of the walls of the fibers (Fig. 32, 33). In the sixth internodes the controls and those treated with the growth substance had thick-walled fibers (Fig. 34, 35, 36). There appeared to be little difference in the number of fibers with these three treatments, but the wall thickening was greater in stems that were treated with the α-naphthaleneacetamide in lanolin.

It has frequently been shown that chemicals can be used to set fruit. It was found that a 0.5 per cent mixture of β-naphthoxyacetic acid could be used to set fruits satisfactorily, but that frequently the paste came in contact with ovaries resulting in misshapen fruit. Therefore the spray method was used at a concentration of 75 milligrams per liter. Cross sections of the nearly mature fruit showed the absence of seed in the

fruits sprayed with β-naphthoxyacetic acid. Hollow fruits sometimes occurred in the sprayed fruit, but occasionally these were also found in the pollinated ones. Young fruits were prepared for a study of the mineral residue in sprayed and pollinated fruits. After microincineration the ash residue in the sprayed and pollinated fruits were compared. There was a like mineral pattern in both sprayed and unsprayed fruits which agrees with reports made by others that the mineral composition as shown by analysis is similar when fruits are set either by pollen or with chemicals.

Alpha-naphthaleneacetic acid is used to thin apple blossoms and also to delay or prevent fruit-dropping at harvest time. The question was raised as to how this growth substance could have seemingly opposite effects. An investigation now under way shows that it has but the one effect of delaying dropping of fruit. The thinning of blossoms is the result of heavy nutritional dropping following heavier early sets induced by α-naphthaleneacetic acid. Therefore, the final result is fewer fruits when the acid is applied at the blossoming stage.

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Formative Effects of

Hormone-Like Growth Regulators

P. W. ZIMMERMAN

FORMATIVE effects may be defined as changes in pattern from that normally resulting from the genetic constitution of the plant under the influence of particular environments. When the environment is more or less constant for a usual habitat, the size and shape of the plant and pattern of organs are said to be normal for a given species. Unusual environments involving temperature, moisture, light, or chemical substances bring about different expressions of so-called normal characteristics. The results may be referred to as formative effects brought about by a given combination of influences. At this time we shall be concerned especially with chemical influences.

There are three groups of chemical compounds which cause modifications in form of plants. These are β-naphthoxyaliphatic acids (13), substituted phenoxyaliphatic acids, and substituted benzoic acids (14). There are other less well-known synthetic compounds and even extracts of plant tissue which have formative influences. In addition to having a formative influence, the naphthoxy and phenoxy groups of compounds have many other hormone-like characteristics. For example, they cause cell elongation, cell division, curvatures, and induction of roots. The derivatives of benzoic acid cause formative effects with little or no cell elongation. 2,3,6-Trichlorobenzoic acid is an exception.

There is need for a single term to cover all substances which have formative effects on plants. The word formagen was proposed by Zimmerman and Hitchcock (15) and used by King (5). Beal proposed the word telomorphic (2), but neither of these words has been widely accepted. I shall, however, use the word formagen at present as a matter of convenience.

The formative influence is not apparent on parts of the plant present at the time the chemical is applied. Only those parts which grow after the plant is treated show formative effects. Old leaves, for example, cannot be reshaped (Fig. 1).

TABLE 1

Molecular configuration and comparative activity for cell elongation involving the tomato plant as test object
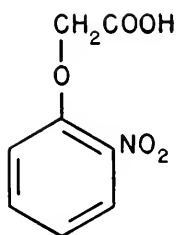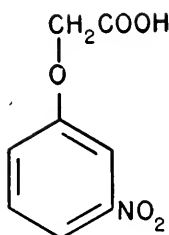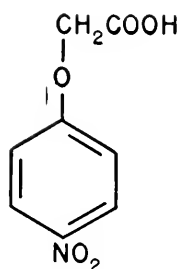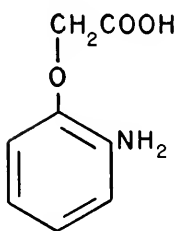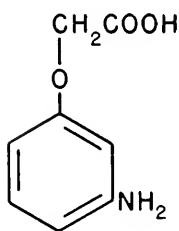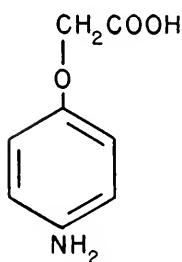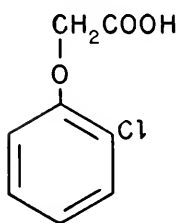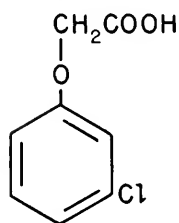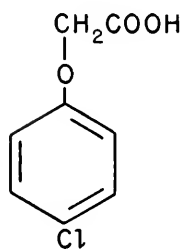
*Inactive for formative effects.
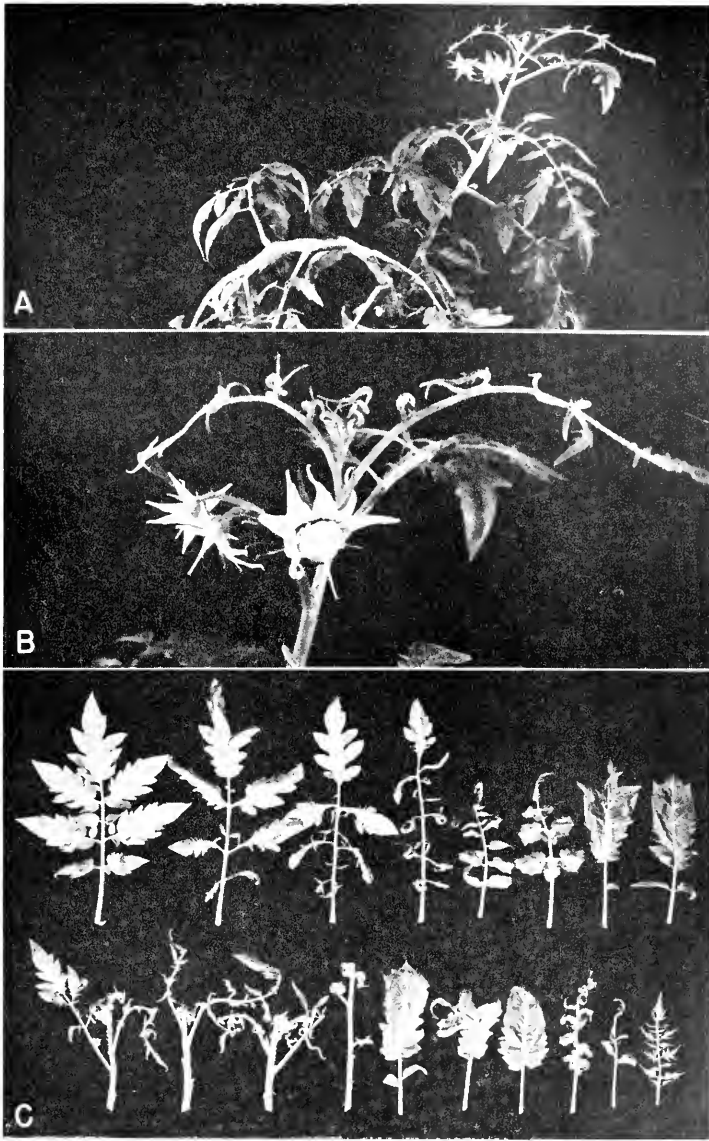
Figure 1. *A*. Y-shaped tomato plant. Left branch treated near middle with *p*-chlorophenoxyacetic acid 20 mg. g. of lanolin. Note modified new leaves and flowers of shoot on right. *B*. Enlargement of "*A*" showing parthenocarpy before flower opened. *C*. Tomato leaves. Upper row from one plant; lower row, modified shoots and leaves taken at random from plants treated with chlorophenoxyacetic acid compounds.

Figure 2. Tomato shoots showing effect of 2,3,5-triiodobenzoic acid (TIB).
A. Tomato stems to show effect of applying 5 mg. of TIB in 50 ml. of water
to soil of a four-inch pot on June 5. Photograph was taken July 17. Left,
control stem with normal flower cluster; middle, axillary flower cluster with
an abnormally long heavy peduncle; right, two axillary flower clusters, one
with an abnormally large flower and long peduncles, and the other with a
short peduncle and unorganized yellowish flower tissue at the tip. *B.* Three
tomato shoots. Left, control with a normal flower cluster and axillary leafy
shoot; middle, one large flower cluster having also two leaves and long peduncle
with several node-like structures; right, axillary flower cluster developed from
vegetative tissue. The flower cluster has also a few leaves. The flowers are
abnormally large and peduncle abnormally long.

The principal modifications induced by active substances are changes in flowering habit, size, shape, pattern, and venation of organs. The blade of the leaf is usually reduced in area, and the veins converge toward the midrib. Clearing of the modified veins, mottling, and other symptoms often cause modifications which may be confused with virus diseases. Leaves often fail to separate from each other, forming large modified organs or cups. Flower buds become tubular where sepals fail to separate, and ovaries frequently develop into seedless fruit. Flowering habit and correlation of organs are modified especially by derivatives of benzoic

TABLE 2

Molecular configuration and comparative activity of substituted phenoxyaliphatic acids involving the tomato plant as test object

SUBSTANCES	EPINASTY, THRESHOLD CONCN., MG./G.	MODIFICATION, THRESHOLD CONCN., MG./G.	PARTHENOCARPY, FAVORABLE CONCN., MG./L.	ROOTING, FAVORABLE CONCN., MG./L.
Phenoxyacetic acid	10	Inactive	Inactive	
2-Chlorophenoxyacetic acid	0.5	0.25	300	
3-Chlorophenoxyacetic acid	0.5	Inactive	50	
4-Chlorophenoxyacetic acid	0.125	0.06	100	1
2,4-Dichlorophenoxyacetic acid	0.015	0.003	5	0.32
2,4,5-Trichlorophenoxyacetic acid	0.06	Inactive	25	0.32
2-Chlorophenoxyacetic acid	0.5	0.25	300	
α-(2-Chlorophenoxy)propionic acid	0.25	Inactive	25	1
α-(2-Chlorophenoxy)butyric acid	0.1	Inactive	50	1
4-Chlorophenoxyacetic acid	0.125	0.06	100	1
α-(4-Chlorophenoxy)propionic acid	0.125	Inactive	25	1
α-(4-Chlorophenoxy)butyric acid	1	Inactive	50	
2,4-Dichlorophenoxyacetic acid	0.015	0.003	5	0.32
α-(2,4-Dichlorophenoxy)propionic acid	0.5	Inactive	10	1
α-(2,4-Dichlorophenoxy)butyric acid	0.5	Inactive	50	1
2,4,5-Trichlorophenoxyacetic acid	0.06	Inactive	10	0.32
α-(2,4,5-Trichlorophenoxy)propionic acid	0.03	Inactive	10	1
α-(2,4,5-Trichlorophenoxy)butyric acid	0.1	Inactive	25	1

acid (Fig. 2). All of the substances having formative influences also cause ovaries of tomato to develop without pollination (parthenocarpy). They vary in effectiveness and practical value. For example, 1 mg./l. of 2,4-dichlorophenoxyacetic acid (2,4-D) is as effective as 500 mg./l. of some of the derivatives of benzoic acid. Though not as effective as 2,4-D, α-(2-chlorophenoxy)propionic acid is recommended for practice because it does not modify leaves.

Physiological activity of a specific type and for a specific species is associated with molecular configuration as a whole rather than any one part of the molecule. A few examples may be given: 2,4-D is one of the most active compounds known for many hormone-like responses, but 2,6-D is practically inactive; 2,4-D and 4-chlorophenoxyacetic acid both have power to elongate cells and modify the leaves and other organs of tomato (*Lycopersicon esculentum* Mill.) plants. The latter compound causes a striking modification of Turkish tobacco (*Nicotiana tabacum* L.) leaves and *Kalanchoe daigremontiana* Hamet et Perrier plants while 2,4-D does not. 2-Chlorophenoxyacetic acid and 4-chlorophenoxyacetic acid cause cell elongation and modification of tomato leaves while the propionic and butyric homologs cause cell elongation but not modification of leaves. 2,4,5-Trichlorophenoxyacetic acid and higher homologs cause cell elongation but not modification of leaves; 2,4,6-trichlorophenoxyacetic acid does not induce cell elongation but modifies leaves. More illustrations of this sort are shown in the accompanying tables.

Physiological activity can be determined at the present time only by biological tests. Both molecular configuration and the genetic constitution of the species are involved. Tomato and tobacco are closely-related species, but 2,4-D modifies only tomato, whereas 4-chlorophenoxyacetic acid modifies both. The mechanism of modification in the plant is complex, and we can only theorize on what combination of factors makes for activity or inactivity.

The recent work of Burton (3) is welcomed as one of the first attempts to determine what happens to the structure of tissue to bring about these odd forms. Using the bean leaflet as a test object Burton worked out the normal structural developments and compared these with chemically induced modifications. It appears that the normal bean leaflet develops a lamina by the activity of a subepidermal marginal meristem, which produced four internal layers of plate meristem. The adaxial (upper) of these layers develops into the palisade layer and the other three

produce the spongy mesophyll. The veins are initiated by divisions of rows of cells in the layer beneath the embryonic palisade. Many intercellular spaces (air) normally appear in the spongy tissue.

Using three substituted phenoxy acids Burton found that these were more or less specific for given structural variations from normal. For example, 2-chlorophenoxyacetic acid inhibited the formation of intercellular spaces in the spongy tissue; 4-chlorophenoxyacetic acid inhibited the activity of the plate meristem (between the veins) and the veins became approximate with continuous parenchyma. 2,4-D brought about

TABLE 3

Molecular configuration and comparative activity of substituted phenoxyaliphatic acids involving the tomato plant as test object

XYLENOXY COMPOUNDS	CELL ELONGATION	FORMATIVE EFFECTS
3,5-Dimethylphenoxyacetic acid	Inactive	Active
α-(3,5-Dimethylphenoxy)propionic acid	Inactive	Active
α-(3,5-Dimethylphenoxy)butyric acid	Inactive	Active
2,5-Dimethylphenoxyacetic acid	Active	Inactive
α-(2,5-Dimethylphenoxy)propionic acid	Active	Inactive
α-(2,5-Dimethylphenoxy)butyric acid	Active	Inactive
3,4-Dimethylphenoxyacetic acid	Active	Active
α-(3,4-Dimethylphenoxy)propionic acid	Active	Active
α-(3,4-Dimethylphenoxy)butyric acid	Active	Active
2,4-Dimethylphenoxyacetic acid	Active	Active
α-(2,4-Dimethylphenoxy)propionic acid	Active	Active
α-(2,4-Dimethylphenoxy)butyric acid	Active	Active

a progressive modification of all leaves developed after the chemical was applied. The latter compound also caused various structural modifications similar to those of both the other acids. Chlorenchymous tissue was usually confined to the margin, and cells without chlorophyll over vascular tissue gave the veins a transparent effect. Burton worked on only one species, the bean. It will be interesting to see how structures of other species respond to the same chemicals.

Modification of flowering habit and correlation of organs induced with substituted benzoic acids are illustrations of special chemical influence. In 1942 Zimmerman and Hitchcock (14) reported that the pattern of leaves, the flowering habit, and correlation of organs were modified by means of 2,3,5-triiodobenzoic acid. They showed that flower clusters

of tomatoes were induced to grow from axillary buds where leafy shoots normally appear and that terminal buds were replaced with flower clusters. The usual flower clusters also developed along the stem. The treated plants lost much of their apical dominance and produced an abnormally large number of axillary leafy branches. It was further pointed out that though benzoic acid was physiologically inactive, the molecule could be activated by the substitution of amino groups or halogen atoms in the ring (7,10,11,12). The positions 2, 3, and 5 of the nucleus appeared to be the most important for substitutions. None of the mono-substituted compounds tested was found active. 2,5-Diiodo-benzoic acid was active for modification and pattern of leaves but did not induce axillary flowers. 2,3,5-Triiodobenzoic acid induced forma-tive effects and also modified the position of flower clusters. 2-Amino-3,5-diiodobenzoic acid was inactive, but 2-chloro-3,5-diiodobenzoic acid was very active resembling 2,3,5-triiodobenzoic acid (11). 2,3,6-Tri-chlorobenzoic acid causes cell elongation, initiates roots, and modifies leaves. The molecular configuration as a whole rather than any part of the molecule appeared to determine physiological activity.

Galston (4) attempted to initiate flowering of soybeans by the use of 2,3,5-triiodobenzoic acid. Though he succeeded in increasing the number of flowers per plant from 32 on the control to 181 on the treated plant, Galston states that 2,3,5-triiodobenzoic acid does not possess florigenetic properties since it will not induce vegetative soybean plants to flower. He further stated that 2,3,5-triiodobenzoic acid caused morphological responses such as shortening of the internodes, loss of apical dominance, epinasty of young leaves, and so on, but that the chemical itself was without auxin activity. The latter conclusion perhaps was drawn from the fact that 2,3,5-triiodobenzoic acid is not active on the *Avena* coleoptile.

Tumanov and Lizandr (8) found that 2,3,5-triiodobenzoic acid re-tarded growth of *Perilla* and caused formative effects. Alfalfa was more sensitive than *Perilla*. These authors found a difference in sensitivity dur-ing short and long days. The treatment caused a variation in the number and size of leaflets in alfalfa. Spraying with weaker solutions, 0.005 per cent, in long days brought about increased yield of alfalfa seed. Flax and sunflower species showed notable changes in growth when treated with 0.01 per cent solution. This concentration also caused peas to branch through stimulation of axial buds and fusing of leaflets. There was, how-

ever, no definite sign that 2,3,5-triiodobenzoic acid could be considered as having florigenetic properties.

Owen (6) treated a number of species with 2,3,5-triiodobenzoic acid and brought about distortions of leaves, stems, and flowers but failed to change any organ from the vegetative to the flowering stage. Avery and Johnson (1) say that the induction of flowers with 2,3,5-triiodobenzoic acid in place of vegetative tissue in tomato strongly suggests that the substance plays the role of flower-inducing hormones in some plants.

The best support for the earlier findings of Zimmerman and Hitchcock was published by Waard and Roodenburg (9). These workers found that the treatment with 2,3,5-triiodobenzoic acid caused the flower cluster to develop at the top of the plant while the vegetative shoot was suppressed. This was considered as a shifting of the correlative relations between the vegetative shoot and flower cluster. They also reported an increase in the number of initiated flower buds and the formation of axillary flower buds when the plants had very few leaves. In fact the illustration shows the flower buds arising from plants with only cotyledon leaves. They concluded that the chemical has the property of starting the process of flower formation and also that it is possible to shorten the vegetative juvenile stage of tomatoes.

In contrast with the case in the substituted phenoxy acids, the *para* position is not an important location in the molecular configuration for active derivatives of benzoic acid. Not all possible substitutions have been made, but from the information at hand the halogen substitutions in the 2, 3, 5, and 6 positions make the molecule more active than the substitution of amino or nitro groups. For example, 2-amino-3,5-dichlorobenzoic acid is inactive while 2-chloro-3,5-diiodobenzoic acid is very active for modification of leaves and induction of axillary flower clusters. 2,5-Dichlorobenzoic acid is especially active for inducing parthenocarpic fruit and modified leaves but does not induce axillary or terminal flower clusters.

Since the effects of 2,3,5-triiodobenzoic acid vary in summer and winter with the rate of growth of tomato plants, it is evident that the substance cannot work alone. It must depend upon materials made by the plant in order to cause vegetative tissue to produce flowers. It is assumed that plant hormones in general require supporting substances produced by the plant to cause well-known physiological responses.

For example, the rooting response after treatment with indolebutyric acid is conditioned by the age of the experimental species, the environmental growing conditions, and the storage of material in the treated tissue.

Repeated experiments with tomato plants verify the earlier reports (15) that 2,3,5-triiodobenzoic acid and 2-chloro-3,5-diiodobenzoic acid cause axillary buds to develop flower clusters instead of leafy shoots. Branches and main tomato shoots were caused to terminate in flower clusters appearing to supplant a shoot growing point. Treated tomato

TABLE 4

Molecular configuration and comparative activity of derivatives of benzoic acid involving the tomato plant as test object

Substances	Cell elongation	Formative effects
Benzoic acid	Inactive	Inactive
2-Iodobenzoic acid	Inactive	Inactive
3-Iodobenzoic acid	Inactive	Inactive
4-Iodobenzoic acid	Inactive	Inactive
2,4-Diiodobenzoic acid	Inactive	Inactive
2,3,5-Triiodobenzoic acid		Active
3,5-Diiodobenzoic acid	Inactive	Active
2-Iodo-3,5-dibromobenzoic acid	Inactive	Active
2-Chloro-5-nitrobenzoic acid	Inactive	Active
2-Amino-5-chlorobenzoic acid	Inactive	Inactive
2-Bromo-3-nitrobenzoic acid	Active	Active
2-Chloro-3,5-diiodobenzoic acid	Inactive	Active
2,3,6-Trichlorobenzoic acid	Active	Active

plants also developed flower clusters in the usual places along the stem, but they frequently had fewer or more than the normal number of flower buds. In fact, internodal, axillary, and terminal flower clusters were similar and were characterized by extremes from short to long, heavy peduncles, and fasciated flowers mixed with small buds. Upon recovering from the chemical influence plants often produced abnormally large flowers (Fig. 3D). Some of the fasciated flowers when pollinated produced a circular ovary resembling a doughnut (Fig. 3C). Such ovaries had sepals both within and around the ovary. Under certain conditions the axillary peduncles had only yellowish cells at the tip, indicating unorganized flower cells.

Figure 3. Tomato plants. *A*. Uppermost portion of a plant showing axillary flower clusters developed from vegetative tissue and terminal flower cluster supplanting the vegetative growing point. The plant was treated on December 22 with 4 mg. of TIB in 50 ml. of water applied to the soil. Photograph was taken the following February 16. *B*. Uppermost portion of a tomato plant treated with a lanolin preparation of TIB 10 mg. /g. of lanolin on December 22. Photograph was taken February 16. The picture shows axillary flower cluster developed from vegetative tissue. The cluster has one large circular flower and an abnormally large number of associated smaller buds. *C*. Tomato cluster. Tubular peduncle with circular ovary induced with TIB 10 mg./50 cc. of water applied to soil January 11. Photograph was taken on March 8. Note sepals within the circular ovary which developed after pollination. *D*. Terminal and internodal flower cluster induced with 10 mg. TIB/50 cc. of water applied to pot January 11. Photograph was taken March 8. Internodal peduncle is branched and has one abnormally large flower.

Some but not all of the substituted phenoxy acids have formative effects when applied to *Kalanchoe* plants. Two striking differences appeared when 4-chlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid are compared. The former causes a change in correlation of organs, hyponasty of leaves, modification of leaf pattern, and pronounced monstrosities at the terminal part of the plant. In contrast, 2,4-D causes epinasty of leaves and root formation of treated stem tissue but no monstrosities. Similar differences appeared when these two chemicals were tested on Turkish tobacco plants. When applied to tomato plants, however, the effects are nearly indistinguishable (14). Such cases as these make it appear that the molecular configuration of the substance must in some way interlock with the mechanism of the species to bring about a given response. The genetic constitution of the species—perhaps the gene—appears to be the determining factor. This is mentioned because tobacco and tomato are closely related species, and yet they respond differently when tested with these substances.

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Practical Applications
of Growth Regulators

Vegetation Control on Nonagricultural Land

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UNWANTED plants are not exclusively a problem of the farmer; almost everyone has a vegetation control problem. Weeds in lawns, gardens, and vacant lots, undesirable woody and herbaceous plant growth on highway, utility, and railroad rights-of-way are a part of our daily existence. Industrial grounds, canals, ditches, lumber yards, oil-tank farms, and military installations all have their particular problems of vegetation control. For factors of safety, health, fire protection, and general efficiency of many operations, as well as for aesthetic values, vegetation must be confined to desired species or kept from growing beyond certain heights.

In the past we have had to resort almost exclusively to mechanical methods of controlling plant growth. True, certain chemical weed-killers have been used to a limited extent for a number of years, but the discovery of the herbicidal value of the chlorophenoxyacetic acids has given us an entirely new concept of the possibilities of controlling vegetation by chemical means. I wish to discuss certain vegetation control problems, to point out how research on herbicides has helped in their solution, and to mention some remaining problems which you as research workers interested in plant-growth control must take the lead in solving.

It has been my privilege to work with several public utilities in the eastern states on right-of-way vegetation control since 1945, soon after the first publications on 2,4-dichlorophenoxyacetic acid (2,4-D). Our first problem was one of formulation. It was soon found that the esters of 2,4-D were more consistent in their action than the salts, particularly on species with a relatively thick cuticle. Apparently the solubility of the esters in leaf wax is an important factor. In spite of the many reports of

desirable results with certain additives to the salts of 2,4-D the esters are largely used today in right-of-way vegetation control because day in and day out they are the most effective formulations we have for the general run of species, regardless of rainfall subsequent to application. It must be remembered that in right-of-way control many crews must operate continuously over a period of several weeks and a formulation or a chemical must be versatile in its action.

Brush regrowth from woody plants that have periodically been cut off, sometimes for many years, constitutes the chief problem on a utility right-of-way in areas of moderate to heavy rainfall. Experience has shown that spraying may be successfully carried out from about the time the leaves are fully developed until late summer. This is not in conflict with the general concept that plants should be in an active state of growth for best results with 2,4-D. Except under very dry conditions such woody plants are growing actively throughout the summer months. Experience has shown that spraying before the leaves have fully developed will often result in inferior kills. This appears to agree with the concept that 2,4-D is most actively translocated from leaves to stems in association with carbohydrate movement.

It soon became apparent in the early work with 2,4-D on woody plants that many species were highly resistant. In the summer of 1946 an old mountaineer in West Virginia told me that the best blackberries he picked that summer were on the right-of-way that had been sprayed the year before. We had eliminated most of the competing woody species. Because blackberries and other members of the genus *Rubus* are so widespread the entire program for a time appeared discouraging. It takes no ecologist to see that eliminating competing plants would enable a species like blackberry to take over an area.

Screening of a number of compounds related to 2,4-D on blackberry and other resistant species was carried out and 2,4,5-trichlorophenoxy-acetic acid (2,4,5-T) was found to be specific for some of them, particularly members of the genus *Rubus*. During the past two years much of the woody plant control on rights-of-way has been carried out with a mixture of 2,4-D and 2,4,5-T. Admittedly this is a shotgun treatment and at times one or the other component might prove best, but until we know more about how species respond and until the spray operators know more exactly the species with which they will have to contend the mixture appears to be desirable. Not all undesirable woody species are

readily killed by this combination of chemicals, but they do provide a practical measure of control. Perhaps further work will uncover compounds specific for those plants that are somewhat resistant to 2,4-D and 2,4,5-T.

Although some use has been made of low-volume, high-concentrate sprays of the esters of the chlorophenoxyacetic acids in oil, most of the right-of-way work is conducted with more dilute sprays applied by power rigs mounted on trucks. On the average better results have been obtained with larger volumes applied under sufficient pressure to wet stems and foliage on the inside of dense growth. Furthermore, a little drift from a high concentrate spray can cause considerable damage to desirable plants adjacent to rights-of-way areas. A little drift from a more dilute spray carries less toxicant and is less likely to cause difficulty. Low-volume spraying of right-of-way vegetation does have a place where limited access by truck and limited water supply makes the use of high volumes applied by power rig impractical.

Spray operators have learned that they must pay attention to wind velocity and frequently skip certain stretches of the right-of-way adjacent to sensitive crops. Most of the instances of damage to desirable plants adjacent to rights-of-way can be traced to carelessness. 2,4-D and 2,4,5-T are not materials to be sprayed indiscriminately and everyone using them must recognize the hazards before he begins. Areas adjacent to especially sensitive crops must often be omitted from the spraying program for that season even though the wind is blowing in the opposite direction at the time of application. There is always the possibility of dust on the leaves of the foliage at the time of application blowing onto the sensitive crop. The fact that some esters of 2,4-D are volatile has frequently been pointed out as a possible cause of the reaction of crops adjacent to sprayed areas. An analysis of the problem of damage to plants off the right-of-way indicates that spray drift is the chief cause of trouble and the blowing of dust a likely cause in some cases. Although volatility may be a factor the writer feels that it has been overemphasized in relation to the other factors.

Although the chief aim of utilities to date has been to control, and insofar as possible to erradicate, woody plants on the right-of-way much nongrass herbaceous vegetation has been killed by the spraying program. From the ecological standpoint it is interesting to note that many rights-of-way which were formerly infested with brushy growth now

are covered with a dense grass sod after two years of spraying. Apparently grass and grass seed were present in sufficient amounts to permit it to become established once the competing woody plants and tall-growing herbaceous species were eliminated. Where species predominate which are stunted but not killed by the available herbicides a mixed grass and shrub community can apparently be maintained without the woody plants growing excessively large.

During the present season there were many thousands of miles of utility rights-of-way sprayed with the chlorophenoxyacetic acids. This is definitely not a one-season program. One treatment will not result in the eradication of many plants. The general practice at present appears to be to spray woody regrowth up to ten feet tall during the summer and then make a second application the next year. Cost of each of these sprays in many cases is no more than the cost of a single cutting with scythes or brush hooks. After two consecutive years of spraying a third year may be skipped and in some cases the fourth year. It appears that a continuous maintenance program will require respraying at intervals of about three years, depending, of course, on rainfall and species composition. The utilities that have been in this program the longest now conservatively estimate that they are cutting the cost of maintaining their rights-of-way in half.

An interesting recent development in vegetation control is the application of 2,4-D and 2,4,5-T to the bark of woody plants and to cut surfaces of stumps (2). Absorption varies with the species and no doubt with many other conditions; however, good kills have been obtained from application during all seasons of the year. Dormant season application offers the possibility of extending operations to make more continuous use of labor and also may make possible the use of these herbicides on rights-of-way in areas where highly sensitive crops preclude summer foliage spray application.

Highway departments have much the same problem with respect to woody vegetation, and their methods of operation are similar. They must use even greater care, however, with respect to plants on adjoining properties because so often highways transverse populated areas. Much of the utility right-of-way spraying is in mountainous country where there are few crops near by. In addition to spray-gun operations for woody plants some highway departments are using spray booms to advantage, treating the entire area from the edge of the road to the

fence line. Where tall-growing or otherwise obnoxious weeds are present 2,4-D and sometimes 2,4,5-T can be used to good advantage for their control.

Railroads like the highways have a brush problem, which heretofore has been handled almost entirely by mechanical cutting. Like the highway departments they must give a great deal of consideration to sensitive crops adjoining their right-of-way, but some railroads, especially those running through the eastern mountains, are applying sprays of 2,4-D and 2,4,5-T from on-track equipment with successful results. Roadbed treatment is primarily with herbicides of the soil-sterilant or contact type. 2,4-D has been used for roadbeds in only a few cases where nongrass species predominated.

The control of water hyacinth in Florida and Louisiana with 2,4-D is a notable example of the success of this compound in solving an old problem. Tremendous sums have been spent in mechanical chopping of water hyacinth in canals. Now airplane application of relatively small amounts per given area are being successfully used. Visitors in south Florida during the past two years have seen visual evidence of the success of this clean-up campaign. Lotus is another emergent aquatic that has been successfully controlled with 2,4-D particularly by the Tennessee Valley Authority as a part of its mosquito-control activities.

The vegetation-control problems of various industries are so varied that they cannot be discussed individually. Undesirable woody growth and tall-growing herbaceous plants can frequently be brought under control or eradicated on industrial grounds by the proper use of the chlorophenoxyacetic acids. This is also true of the tremendous areas given over to our military establishments.

The use of 2,4-D for weed control in lawns and recreational turf areas is almost too well known to warrant comment. It is of interest that white clover and other legumes that are sometimes undesirable components of turf may be more readily killed by 2,4,5-T than by 2,4-D. In this connection the writer has frequently observed a greater response from semiresistant plants, such as white clover, during hot weather than during the cool seasons of the year.

Poison ivy, one of our most undesirable plants in recreational areas is also more susceptible to 2,4,5-T than to 2,4-D. The difference between these two chemicals is often the difference between a high percentage of eradication and a temporary reduction in top growth.

New chemicals other than the chlorophenoxyacetic acids are becoming available for vegetation control purposes. Possibly one of the most interesting of these is sodium trichloroacetate (sodium TCA) which has a herbicidal effect on many grasses (1). It also appears to be promising for killing cacti and palmetto.

Although relatively large amounts of this chemical are required for a high degree of grass-kill smaller amounts have a practical growth-controlling effect. For example, on northern species such as quack and blue grass twelve to fifteen pounds per acre of sodium TCA applied when the infloresence is first emerging has eliminated flowering and retarded growth for several weeks. When applied as a spray with 2,4-D many nongrasses can be killed and the growth of grass controlled in one operation. This technique appears valuable wherever mechanical mowing cannot be carried out, such as along ditches and around highway guard rails. Combined with the phenolic contact sprays sodium TCA has shown much promise for general weed control on railroad beds.

The action of this chemical, which I am taking the liberty of calling a growth-regulating substance, is little understood. The immediate foliage-burning effect appears to be independent of the systemic effect which with quack grass occurs only after root absorption takes place. In my own work far more grass killing has resulted when the chemical was applied to the surface of newly plowed ground than when grass foliage was sprayed. Obviously soil moisture relations are important with a chemical that is largely absorbed through roots. At times considerable dormancy of grass buds occurs following treatment without a lethal effect. It is hoped that more physiologists will study the action of this new herbicide. It seems likely that many of the variations in field results are related to the physiologic condition of the grass as well as to soil moisture and rainfall.

No doubt we are on the threshold of many further developments in chemical vegetation control. The recent report on maleic hydrazide (3) indicates that growth control without lethal action has definite possibilities for many kinds of plants. Such chemicals could be of great value to highway departments who must maintain turf at a reasonable height and also to public utilities who have a tremendous tree-trimming problem. Needless to say such chemicals would be of interest to all of us who have a weekly session with the lawn mower.

Many problems in vegetation control remain unsolved. We need

growth-regulating substances more efficient than 2,4-D or 2,4,5-T for the control of many species such as ash, smilax, and certain oaks and maples. Although existing chemicals have some effect on cattails, bracken fern, and horsetail we need something more efficient for their control.

Translocation of 2,4-D and 2,4,5-T to the root systems of some weeds is inefficient, as is the case with milkweed and leafy spurge. Better herbicides or improved knowledge of how to use our present ones is needed for such plants. We need chemicals with specific growth-controlling action but without lethal effect for use on trees and shrubs as well as on grasses and certain herbaceous plants. There is need for a chemical for the control of all plant growth on railroad beds and other areas where no vegetation is wanted. Possibly a nonselective herbicide of the growth-regulating type is not beyond the realm of possibility.

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Differential Responses in Crop Plants

R. S. DUNHAM

ONE of the most important characteristics of 2,4-dichlorophenoxy-acetic acid is its selective action. Although the reasons for this selectivity are not fully understood, it has been the basis for a wide practical use. Selective action becomes a distinct advantage because it permits the spraying of certain weeds growing in tolerant crops. Studies of the practical application of selective herbicides are necessarily concerned with the reaction of the crop as well as the weeds.

Research in this phase of investigation soon showed that not only were there differential responses of certain species classed as weeds and others grown as crops but that there were different reactions among crop plants and, even further, that varieties and strains of the same crop might show varying tolerance or resistance to applications of herbicides. Later it was learned that the same variety or strain may be influenced in reaction by environmental conditions. It is the purpose of this paper to discuss such differential responses among field-crop plants, particularly the small grains, flax, and corn. The different phases of the problem will be discussed separately by reviewing the pertinent literature and presenting short summaries of new information from unpublished experiments of the writer and co-workers.

SELECTIVE ACTION OF 2,4-D

Species and varietal differences.—Norman (39) points out that selectivity is only an apparent one based on different degrees of susceptibility to a particular dosage applied in a particular way, and Crafts (10) states that even tolerant plants succumb provided the concentration is high

enough. This conception is unquestionably true. Nevertheless, the differences in response of plants within certain limits of dosage, time of application, and environment are large enough to make possible the killing or severe injury of certain undesirable species in the presence of crop plants. Generally the monocotyledons have been more resistant to 2,4-D than the dicotyledons, but there are several notable exceptions in each group. Among the monocotyledons certain strains of the bent grasses and buffalo grass have proved susceptible. Among the leguminous crops Buchholtz (4) found red clover more tolerant than alfalfa, and Willard and Shaw (48) found Ladino white clover and common white clover more tolerant than alfalfa, red clover, sweet clover, alsike clover, and lespedeza although all species could be classed as relatively susceptible. Because of their susceptibility to 2,4-D, the spraying of legumes with this herbicide has commonly been considered too hazardous, but thousands of acres of flax, also a dicotyledon, have been sprayed successfully.

Marked varietal differences in response to 2,4-D both in monocotyledons and dicotyledons have been observed by many investigators. Dunham and Tandon (16) found large varietal differences in tolerance of flax to 2,4-D. An application of four times the amount that reduced the yield of Crystal and B5128 did not injure Redwing. Similar or even larger differences exist in corn. Buckley (7), Holden et al. (26), Jugenheimer et al. (27), Lee (31), Leng and Slife (33), Miller (37), Rossman and Staniforth (41), and Viehmeyer (46) found wide differences in response to 2,4-D among inbreds, among single crosses, and among double crosses. Elder and Davies (18) reported varietal differences in sorghum. Derscheid et al. (12) found no significant reduction in yield among nine oat varieties but did find differences among barley varieties. Seven varieties of spring wheat responded in a similar manner to 2,4-D according to Helgeson et al. (25). Sexsmith (42) noted no differences among six varieties. Albrecht (2) concluded that bent grass strains vary considerably in their tolerance to injury from 2,4-D.

Some environmental effects.—Differences in reaction to 2,4-D may be very marked among varieties and strains but they are influenced by the dosage, the time of application, and environmental factors. Dunham and Robinson (15) sprayed ten varieties of flax with 4 oz. of triethanolamine and 1.3 oz. of butyl ester per acre and reported that all varieties responded alike at these low dosages. Dunham (13) states that varieties of flax differ most widely in their response to 2,4-D: (a) when the ester

is used, (b) when rates of amine or sodium salts are heavier than recommended, and (c) when applications are made in bud and bloom stage. There is evidence also that both temperature and sunlight are influencing factors (28,34,47). Arakeri and Dunham (3) studied the relation of some environmental factors to the pre-emergence treatment of corn with 2,4-D and concluded that out of all the factors studied, the most important were water, soil type, pH, and organic matter content of the soil; less important was time of application, and least important were depth of planting and dosages.

Stage of growth and selectivity.—The stage of growth of a plant at the time it is sprayed has been recognized as an important factor by many investigators. Commonly it has been reported in terms of height. More recently an attempt has been made by Dunham *et al.* (14) to describe the stage of small grains and flax in terms of morphological development. The "tiller," "shooting," and "boot" stages of wheat and the cotyledon, true leaf, and stem elongation stages of flax are illustrated.

Derscheid (11), in summarizing the abstracts of 27 investigators who worked with 2,4-D on spring wheat, oats and barley, states: "These data indicate that all three crops are less tolerant at early 3-leaf and 5-leaf stages of growth than after they have become fully tillered. The most susceptible period, however, is when heads are emerging from the boot." Elder (17) reviewed the data on winter wheat and states that it "is more resistant to 2,4-D in early spring when fully tillered or in early joint stage and more susceptible when treated soon after planting in the fall months. The boot to heading stage is a susceptible period." Dunham *et al.* (14) state that information relative to flax is not so clearly established as for the small grains, but apparently the most susceptible time is from bud formation to bloom. They also advise the farmer to avoid spraying when the stem is rapidly lengthening.

There is increasing evidence that susceptibility is closely related to periods of rapid growth in the plant. Conflicting results with corn when described in terms of the height of the plant can be explained on the basis of rapidity of growth. Lee (30) reports that: "Experiments conducted in both 1947 and 1948 indicate that small corn is more susceptible to damage than larger corn. This year (1948) in Indiana large corn, 12 inches or more in height, was more easily damaged than small corn. The reason was apparently because of the difference in growing conditions."

Paatela (40) made intensive studies of the relation of increase in height of flax to its susceptibility to methoxone and 2,4-D. In experiment 1, flax plots sown on the same day were sprayed over a period of six weeks as they reached seven stages of development. Thus treatment I was made when the flax was in the cotyledon stage; II, when 4.6 cm. tall; III, 7.8 cm. tall; IV, 12.5 cm. tall; V, 17.1 cm. tall; VI, in bud; and VII, in flower.

In experiment 2, stages I, II, V, and VI were obtained by sowing the flax at successive dates. All plots were sprayed on the same day. Since the date of spraying was July 1, growth conditions were favorable for more rapid growth of flax in all stages as compared to experiment 1. This fact was determined by height measurements of unsprayed flax for a period of six days following the spraying of the remaining plots. Injury was markedly greater in experiment 2 than in experiment 1, with one exception, even though the flax was in the same stage of development in each case. The one exception was the flax sprayed in the cotyledon stage. Data illustrating this result were selected in cooperation with Paatela and are presented in Table 1.

In a more recent study at Minnesota, Paatela and Dunham grew three varieties of flax under conditions conducive to slow growth in one instance and rapid growth in the other. The following treatments were used: 1) Flax was grown 3–4 inches tall with 12 true leaves at approximately 50° F. and sprayed with (a) methoxone and (b) amine salt of 2,4-D. One half the population remained in the cool room; the other half was removed to the greenhouse at time of spraying. 2) Flax was grown to the same stage as in 1 at high (approximately 85° F.) temperatures in the greenhouse. One half the population remained in the greenhouse; and the other half of the population was removed to the cool room at time of spraying. Response was measured by the bending and twisting of the stems, a characteristic reaction of flax to the growth-regulating herbicides. Both the number of affected plants and the degree of epinasty were recorded. The number of plants multiplied by the degree of bending as indicated by a scale of 0 to 3 was divided by the total number of plants. The resulting decimals are the data reported in Table 2. Differences appear small when expressed in decimals until comparisons are made. Thus 6 oz. of methoxone on Dakota started in the warm room and moved to the cool room caused 8 times as much bending as when the plant was grown continuously in the cool room (0.8 and 0.1). It is

apparent that all three varieties showed the greatest response when started in the warm room and transferred to the cool chamber and the least, even at high dosages, when started in the cool chamber whether they remained there or not. Varietal differences are also apparent in this treatment.

TABLE 1

Comparison of increase in height of untreated flax and yield of seed from flax sown the same day and sprayed at four stages of growth (Experiment 1) versus untreated flax and flax sown on four dates and sprayed on the same day (Experiment 2).

STAGE OF PLANTS AT TIME OF TREATMENT	INCREASE IN HEIGHT OF UNTREATED PLANTS 6 DAYS AFTER TREATED PLANTS WERE SPRAYED	YIELD OF SEED WITH (UNTREATED = 100)	
		METHOXONE	SODIUM SALT OF 2,4-D
	cms.		
Cotyledon (Exp. 1)	1.8	117	66
" (" 2)	2.5	110	86
4.6 cm. (Exp. 1)	3.5	115	105
5.3 " (" 2)	7.4	102	92
17.1 cm. (Exp. 1)	12.6	105	98
19.2 " (" 2)	15.0	82	91
Bud (Exp. 1)	6.6	105	52*
" (" 2)	12.3	81	20*

*Morpholine salt of 2,4-D.

An experiment now in progress at Minnesota by Shulstad, Dunham, and Heggeness indicates that the rate of increasing height differs among flax varieties; that this rate differs for the same variety when sown at ten-day intervals; and that the order of varieties ranked according to rate of increase in height varies with different planting dates. The experiment has not been completed, but there is also evidence that varieties rated on the basis of tolerance to 2,4-D under a given set of growing conditions do not necessarily maintain that order under a different environment.

It appears from all the evidence available that rapidity of growth is an important factor in determining the susceptibility or tolerance of flax

TABLE 2

Response of Minerva, Dakota, and Redwing flax to applications of methoxone and 2,4-D made under variable growing conditions.

TREATMENT	RESPONSE*		
	REDWING	DAKOTA	MINERVA
Started in cool room			
Remained in cool room			
6 oz. methoxone	0.1	0.1	0.0
6 oz. 2,4-D	0.1	0.0	0.0
12 oz. methoxone	0.1	0.2	——
12 oz. 2,4-D	0.0	0.1	——
18 oz. methoxone	0.1	0.3	
18 oz. 2,4-D	0.1	0.1	
Started in cool room			
Removed to warm room			
6 oz. methoxone	0.1	0.2	0.1
6 oz. 2,4-D	0.0	0.1	0.0
12 oz. methoxone	0.3	0.2	——
12 oz. 2,4-D	0.1	0.2	——
18 oz. methoxone	0.3	0.4	
18 oz. 2,4-D	0.2	0.4	
Started in warm room			
Moved to cool room			
6 oz. methoxone	0.4	0.8	0.7
6 oz. 2,4-D	1.0	1.1	1.6
Started in warm room			
Remained in warm room			
6 oz. methoxone	0.1	0.2	0.1
6 oz. 2,4-D	0.4	0.2	0.8

*(Number of plants affected) x (scale of 0 to 3 for degree of bending) divided by (total number of plants).

to 2,4-D. Although varietal differences exist, they are not constant under all conditions.

Dunham et al. (14) have pointed out the association of rapid growth and stage of plant. Thus, the small grains commonly grow slowly until well tillered, a stage relatively tolerant. The following period of jointing or shooting is one of rapid elongation under favorable growing conditions and is generally a more susceptible stage. Likewise in flax the period of stem elongation is commonly avoided in spraying for, under favorable conditions, it is one of rapid growth.

Numerous investigators have reported reduced yields from plants

sprayed when flower development is active. Thus the boot and heading stage of small grains, the bud and bloom stage of flax, and the tasseling stage of corn have been singled out as particularly susceptible periods. It is questionable whether rapidity of growth or development is very closely associated with susceptibility in these stages. It is quite probable that spraying at these times affects the flower development so that the injury is directly expressed in yield of seed.

Morphological responses of plants to 2,4-D.—The reaction of crop plants sprayed with 2,4-D is often characteristic. Morphological changes as described in the literature are as follows:

> Wheat exhibits club-shaped spikes, irregular arrangement of spikelets, branched rachis, 2 spikelets per rachis node, fused glumes, thickened culm, and chlorosis. (See 20,21,32,42).
> Oats show onion leaf, blasted spikelets, late tillering, and interference with heading. (See 8,23,44).
> Barley shows extended internodes on rachis, round internodes, 2, 3, or more kernels at each rachis node in 2-row varieties, naked kernels, multiple awns, spikelet groups in 2-row resembling 6-row type (1).
> Flax shows bending and twisting of stem, twin bolls, fused leaves, swollen stems, excessive branching, death of the central stem, and chlorosis. (See 16,45).
> Corn shows stalk curvature, stalk brittleness, lodging, fasciation of brace roots, and onion leaf. (See 6,7,22,31,43).

These abnormalities are not necessarily permanent. Frequently plants recover from them with no detrimental effect on yield (6,7,25,36,44,45). Delay in maturity, and reduction in height are commonly mentioned in the literature as effects of 2,4-D application. Tandon (45) points out that the delay in maturity of sprayed flax is greater at the final bloom stage than when ripe and that at practical rates of application this delay in maturity "is not of much practical consequence since the sprayed flax was not more than 2 days later than the check." Commenting on the frequent reports of serious delay in flax, Dunham has pointed out that two possible explanations for these contradictory results cannot be tested because of insufficient pertinent information in the reports submitted. Delay as reported may have been measured at bloom, rather than at maturity. Flax ripening too late in the season

may prolong the process abnormally and any delaying effect of treatment might be exaggerated (13). Tandon (45) made fortnightly measurements of height in seven varieties of flax sprayed with 0, 4, 8, and 16 oz. of sodium salt, amine salt, and ester of 2,4-D. He concluded that up to 8 ounces there is little reduction in height, and as the crop advances towards maturity even the little reduction noticed at earlier stages is practically eliminated. Paatela (40) has also reported that before the final height (of flax) was recovered, the average height of treated plants increased (more than the untreated) and the increase was greatest for plants treated with the highest concentration.

Chemical and physiological responses of plants to 2,4-D.—The differential response of crop plants to applications of 2,4-D may be expressed in factors affecting quality. Most of the work reported has dealt with protein content of wheat and with the oil content and iodine number of the oil in flax. Helgeson *et al.* (25) and Mitchell and Linder (38) report increases in the protein content of sprayed wheat, but the increase was associated with a reduced yield, an association that would normally be expected. Corns (9) obtained an increase in protein of barley when yields were reduced by more than five bushels. Erickson, Seely, and Klages (19), however, report an increase in protein of wheat without a corresponding decrease in yield.

The effect on the oil content and iodine number of the oil in flax has been investigated by Tandon (45), Klosterman and Clagett (29), and Paatela (40). Recent studies of the writer and co-workers will be outlined briefly. Tandon found a distinct differential response among seven varieties to the amine and sodium salts applied at 4, 8, and 16 oz. per acre. The ester of 2,4-D at these rates reduced both oil percentage and iodine number in all varieties tried except the oil in B5128 at 4 oz. per acre. Paatela reported a reduction in oil up to 2.3 per cent when the flax was sprayed with the morpholine salt of 2,4-D in the bud stage and a reduction in the iodine number when treated in the cotyledon stage. Square yard samples were obtained by Klosterman and Clagett from sprayed and unsprayed portions of four fields. The spray was applied at 0.175 pounds of 2,4-D acid per acre in the form of the alkanolamine salt. The variety Dakota was grown on two fields and Minerva and Sheyenne on one field each. Stages of growth varied between fields at time of spraying. No significant differences were found in oil percentage; the iodine number of sprayed Dakota was significantly higher than that of

the unsprayed, while that of unsprayed Minerva was higher than that of sprayed. The authors conclude that this formulation at the dosage used "is not detrimental to the value of the flax crop."

Further investigation has been completed recently by Dunham and Robinson. Koto flax sown in rod rows was sprayed in the 2-inch stage, the prebud stage, the late bud stage, and the full bloom stage with 4, 8, and 24 oz. of the sodium salt, the amine salt, and the ester of 2,4-D. The data for the sodium salt are reported in Table 3. It is clear that the relatively tolerant Koto variety was adversely affected by the treatment since differences in percentages of oil were significant at the 1 per cent point and in iodine number at the 5 per cent point according to the t test.

Largest reductions in oil percentage resulted from spraying in prebud and late bud stages, the first stage representing the approximate end of vegetative growth. Differences from the 4 oz. treatment may not be significant except in late bud and full bloom stages, but there is a reduction in all instances.

Likewise the iodine number was adversely affected in general. The two exceptions among 12 paired comparisons are the 4 oz. and 24 oz. applications at prebud.

In the 1948 variety test at Minnesota the percentage of oil in Minerva, Victory, and B5128 was reduced 2.37 per cent, 0.56 per cent and 0.91 per cent respectively with only 1.3 oz. of 2,4-D acid supplied as butyl ester per acre.

To the pure-seed producer the effect of 2,4-D on germination of seeds produced on sprayed plants is of vital concern. Buchholtz (5), Derscheid (12), and Goodwin et al. (24) report the viability of oats unhurt. Elder (17) reports no injury to winter wheat seed. Helgeson et al. (25) stated that "germination of grain (hard red spring and durum wheats) was reduced by ester treatment in the boot stage only." Dunham and Robinson sprayed corn at five stages of growth and oats and barley at two stages each without injuring germination of the resulting seed. Tandon's (45) data show "that not even those varieties (of flax) which were susceptible to 2,4-D in other respects showed any reduction in the viability of seed." Further work at Minnesota found this to be true only when the flax was sprayed before late bud stage. Marth et al. (35,36) report no injury to the viability of Kentucky bluegrass or timothy seed from treated plants.

TABLE 3

Oil content and iodine number in Koto flax sprayed with sodium 2,4-D at various dosages and dates.

Stages of flax	Amount of 2,4-D	Unsprayed	Sprayed	Difference
		Oil per cent		
2 inches	4 oz.	32.39	32.20	.19
	12	32.27	31.79	.48
	24	32.48	31.93	.55
Prebud	4	32.94	32.55	.39
	12	33.40	31.82	1.58
	24	33.04	31.70	1.34
Late bud	4	32.50	31.91	.59
	12	32.56	31.82	.74
	24	32.33	31.48	.85
Full bloom	4	33.32	32.49	.83
	12	32.58	32.40	.18
	24	31.96	32.42	− .46
Average		32.65	32.04	.61
S. E. unsprayed plots		.44		
		Iodine number		
2 inches	4	167.6	167.0	.6
	12	167.3	164.8	2.5
	24	167.2	164.9	2.3
Prebud	4	165.9	168.3	−2.4
	12	167.4	164.1	3.3
	24	160.4	163.5	−3.1
Late bud	4	166.5	166.3	.2
	12	166.9	161.3	5.6
	24	165.7	158.3	7.4
Full bloom	4	167.7	158.3	9.4
	12	167.9	162.8	5.1
	24	167.3	166.9	.4
Average		166.5	163.9	2.6
S. E. unsprayed plots		2.04		

Differences in percentages of oil were significant at the 1 per cent point and in iodine number at the 5 per cent point according to the t test.

Summary

This paper discusses the response of crop plants especially small grains, flax, and corn to the applications of growth-regulator herbicides, particularly 2,4-D. The work of numerous investigators is cited, and original research not yet published is presented. The following statements summarize the discussion.

1) The selective action of 2,4-D makes possible the killing or severe injury of certain undesirable species in the presence of crop plants. Differences in reaction to 2,4-D occur among species and among varieties and strains of the same species.

2) These differences may be influenced by dosage, time of application, and environment.

3) The rapidity of growth during the vegetative phase of plant development and/or the stage of flower development at the time of application are important factors influencing the results from using 2,4-D or methoxone.

4) The morphological reaction of crop plants is often characteristic. Some abnormalities resulting from spraying with 2,4-D are listed. It is pointed out that plants frequently recover completely from such initial effects.

5) Increase in the protein content of wheat and barley and reduction of the oil content and iodine number of the oil in flax have been reported. Varietal differences have been found to this reaction in flax. The viability of seed from crop plants sprayed with 2,4-D at stages before bud has not been injured.

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Growth Substances in Relation to the Production

of Tree Fruits

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THE propensity of many of the plant growth substances to influence abscission of flowers, fruits, and leaves has been used to considerable practical advantage in horticulture, particularly in relation to tree fruits. Strangely enough, the growth substances may act not only to delay abscission but also to implement it, and both phenomena have found horticultural application. The answer to this seeming paradox is by no means clear, although it is perhaps to be explained by the mode of abscission encountered, which differs in different kinds of plants and in different organs of the same plant. This thought will be referred to in more detail as this discussion develops.

This review of accomplishments with plant hormones will be restricted to tree fruits and will treat of control of mature fruit drop and of fruit thinning and its opposite corollary, fruit set, with brief mention also of several problems which call for investigation. A mere review of literature would seem inadequate to the occasion if it did not treat of the subject objectively, attempting to trace the advances made to the present status of accomplishment, pointing out the failures and the present blind spots in our understanding, and developing some rationale for the approach to new achievements in the light of our present knowledge, limited as it is.

CONTROL OF PREHARVEST FRUIT DROP

Apples.—The possibility of using growth substances to control the drop of apple fruits was suggested by the observed effect of these compounds in causing the persistence of leaf petiole stubs on mis-

cellaneous cuttings that had been treated to stimulate rooting. Other instances were also noted of delayed abscission of various treated organs, particularly floral parts. Since the first report of control of apple drop in 1939 by Gardner, Marth, and Batjer (16) there have been not less than seventy-five scientific publications dealing with hormone chemicals in relation to fruit drop. As a result of these various reports hormone sprays have become a standard orchard practice for apples and pears in most sections where these fruits are grown, in many cases being applied by airplane over large acreages. It should be noted that at the time the idea was conceived plant-hormone chemicals were being produced in minor quantities and consequently were sold at prices that would make their use for orchard spraying appear to be fantastic. It has been the history of most synthetic biological compounds, however, that chemical know-how coupled with sufficient demand has resulted in great price reductions. Accordingly, a plant investigator should not discard, untried, his ideas for practical applications simply because of the current price of the compound under consideration.

Little would be served by attempting to review all of the papers relating to apple drop control. The first detailed report of the original work (17) established the effectiveness of α-naphthaleneacetic acid (NAA) and its amide at concentrations of 5 to 10 ppm. Many of the subsequent reports served to confirm the early findings and to extend the results to additional varieties and conditions. Omission of specific mention of each of these numerous papers in no way reflects on their importance and helpfulness. A number of reports have served to demonstrate the usefulness of various carriers for the hormone compounds and also methods of application. Still others have given techniques for screening compounds for effectiveness and have furthered the knowledge of penetration and movement of these substances within the tree.

Despite efforts to find more effective or more adaptable compounds for drop control, NAA and its amide and salts remain the most useful. In searching for more effective compounds Batjer and Marth (3) found that 2,4-dichlorophenoxyacetic acid (2,4-D) applied to Winesap apples extended the effective period greatly beyond that of NAA, but that it did not take effect as quickly. The results were so exceptionally good on Winesap that many other varieties were subsequently tried by Batjer and Thompson (4) and Harley et al. (19,20). Unfortunately, the only

apple varieties reported thus far to be appreciably affected by 2,4-D
sprays are Winesap, Stayman Winesap, Kendall, and Bonum, the last
two being minor varieties. Apparently the differential effect of 2,4-D
on different species of plants, which is the basis of its usefulness as an
herbicide, extends even to varieties.

Those workers interested in attempting to explain the differential
herbicidal effect of 2,4-D might well ponder the results of Edgerton and
Hoffman (14), who introduced a solution of this compound into the
transpiration stream of McIntosh apple trees. Numerous attempts to
control fruit drop of McIntosh variety by 2,4-D sprays have been
uniformly unsuccessful, but a very definite effect was secured by the
injection method. Although abscission of both fruit and leaves was de-
layed, no injury to the trees was reported.

While sprays of 2,4-D are effective in controlling drop of a few
varieties, its use is not without hazard in apple and pear orchards. For
best results it should be applied earlier than NAA; but the earlier it is
applied the more danger there is of damaging holdover effects which may
be expressed in deformed leaves and shoots the next spring. Marsh and
Taylor (27) described some severe damage to susceptible varieties of
apple from 2,4-D residue in a central spray system which had been
utilized for weed control and subsequently used for a late spraying
of the trees with summer oil. The persistence of the effect of 2,4-D is
sometimes remarkable. Moon, Regeimbal, and Harley (32) reported
a case in which Stayman Winesap, sprayed in August, 1946, showed
appreciable drop control in the following year's crop picked in October,
1947.

Citrus Fruits.—Preharvest drop of oranges and grapefruit, unlike that
of apples and pears which in general occurs during the few weeks prior
to time of picking maturity, may straggle along for many weeks. This
situation is due in part to the fact that citrus, not being a starchy fruit,
has no definite physiological maturity stage. Once ripe enough to eat,
its picking may be, and usually is, delayed for weeks or even months
in order to catch a favorable market. Thus the cumulative loss from
droppage may be appreciable, rarely amounting to less than 10 per cent
of the crop and often much more. Certain varieties, such as Pineapple
and Temple oranges, may also exhibit a wave of heavy droppage in the
latter part of their season, in which most of the fruit drops within a few

days' time. Tangerines, on the other hand, adhere tenaciously, and it is only with difficulty that mature fruit of any stage can be pulled intact from the tree.

The response of citrus fruits to sprays of naphthaleneacetamide is quite in contrast to the response of apples. Gardner (15) found that 10 ppm. of this compound had no effect on Pineapple oranges but that 100 ppm. reduced drop markedly if applied early in the harvest season (November) and was still effective 12 weeks later when the fruit was harvested in February. When applied in January even this high concentration was without effect. Most citrus growers would prefer to take the chance that they will harvest their crop ahead of heavy droppage rather than invest in the cost of such a concentrated spray, and for this reason, in part, the naphthalene compounds are not used for citrus.

In California, Stewart and Klotz (45) reported appreciable reduction in the preharvest drop of Valencia and Washington Navel oranges from 2,4-D sprays applied in early summer. Marsh grapefruit, in experiments by Stewart and Parker (46), also responded but apparently not as satisfactorily as oranges. At the highest concentration (225 ppm.) some of the young grapefruit on the trees at time of spraying (not the mature crop for which the sprays were applied) developed quite abnormally, having cylindrical shapes, thick rinds, many prominent rudimentary seeds, and even navels. The oranges responded similarly at high concentrations (45). From numerous reports by Stewart and his coworkers it appears that low concentrations, 5 ppm. for example, are nearly as effective as 25 ppm., at which concentration and above occur increasing injury to the tree, abnormal fruits, and abscission of young fruits of the new crop. This reduction in the quantity of fruit might well account for the slightly larger fruit sizes of oranges reported by Stewart (44).

In Florida, Gardner (unpublished results), using the sodium salt of 2,4-D on several citrus varieties in October at 10 ppm., did not secure as outstanding control of drop as that reported in California. The Pineapple oranges picked 12 weeks after spraying dropped half as much fruit during that period as the controls. The sprays on Valencias and on Marsh grapefruit were somewhat less effective and, in the case of grapefruit, the effect did not persist as long as with oranges. "Seedling oranges," which approach a varietal status in Florida because of the high incidence of nucellar seedlings, failed completely to respond to the 2,4-D. It appears that here again is an instance of differential varietal

susceptibility, as in the case of apples. A great deal more work needs to be done under Florida conditions and with Florida varieties to establish the most effective safe concentrations and times for application. In the Florida drop control experiments the sprays have thus far been applied in the late fall months not only because this is near the beginning of the drop period, but also because the trees are not flushing new growth and do not normally do so until February and can therefore withstand a higher concentration without injury than at other periods. Moreover, sulphur sprays and dusts for rust mite control are commonly applied at this time, and it appears entirely feasible to include 2,4-D at no extra expense other than its insignificant cost. The results thus far indicate, however, that the presence of wettable sulphur moderately reduces the effectiveness of the 2,4-D, although it is possible that this difficulty may be overcome simply by increasing the amount used. The low cost of this material and the possibility of including it in pest-control sprays are important assets favoring its wide adoption for citrus fruits.

Pears.—Passing reference has already been made to the drop of pears, but since this fruit responds to hormone sprays so readily, it appears desirable to review briefly the present status of its drop control. Summer pears for the fresh fruit market are harvested prior to full maturity, and thus a drop problem is usually not of great moment. Canners, on the other hand, need to have the fruit more nearly ripe, and the delay in picking for this purpose and for increased size results in appreciable amounts of grounded fruit. Apparently Strickland *et al.* (47) in Australia were the first to report pear drop control by hormone sprays. Their treatments with 20 ppm. of naphthaleneacetamide in three applications have since been shown to be much more extravagant than necessary. Davey and Hesse (12) obtained appreciable control in Bartlett variety with both NAA and its amide at 5 and 10 ppm. They make no mention of injury from NAA at the higher concentration, although Batjer *et al.* (6) reported a yellowing and premature drop of considerable foliage from this concentration. These last investigators obtained effective drop control of Bartletts with 2.5 ppm. of either NAA or 2,4-D. Concentrations of 2,4-D higher than 2.5 ppm. (5 and 10 ppm.) caused yellowing of old foliage, injury to buds, and malformation of new fruit and foliage in the following season proportional to the increase in concentration. Overholser *et al.* (35) mentioned that Bosc variety, as well as Bartlett, responds to NAA; but these workers did not try 2,4-D.

Peaches and Apricots.—Peaches and apricots delayed in picking to attain proper maturity for canning are subject to considerable drop in certain areas. Hesse and Davey (21) found that the Stewart apricot responded to both NAA and its amide but that with Elberta peaches the response was so slight as to hold no commercial advantage. Effective sprays for peach drop control would be a boon to the canning-peach industry.

Effect of preharvest sprays on fruit maturity.—Space will not permit a review of the work on the effect of growth substance sprays on fruit maturity, but the problem is of such importance in fruit storage that at least brief mention should be included here. It now seems quite certain that growth substances can and do hasten maturity of apples and pears on the tree and affect their subsequent storage life. The extent of this effect depends on the compounds used and their concentration, as well as on the length of delay in harvest made possible by their use. Citrus constitutes a notable exception, perhaps unfortunately, for in this case a hastening of maturity might have important advantages in marketing. The results of Blondeau and Crane (9) in hastening the maturation of Calimyrna figs from a normal 120-day period to 60 days by sprays of 2,4,5-trichlorophenoxyacetic acid indicate that important advantages in time of ripening might be gained with certain fruits.

Some Factors Influencing Effectiveness

Methods of application.—Dust applications, assuming equal effectiveness, have advantages over spray applications on the basis of lower labor costs and more rapid coverage, particularly important in case of large acreages. Hoffman, Edgerton, and Van Doren (23,24) reported that under favorable dusting conditions NAA incorporated in a talc dust was equivalent in drop control of McIntosh apples to roughly the same quantity of the compound applied as a spray. Southwick (39,40), on the other hand, using the same concentrations and the same variety, did not find dusts to be equal to sprays. Unfavorable conditions for dusting are more apt to occur than for spraying, and this may explain the lack of complete agreement. In subsequent work Southwick's results with dusts were more nearly the equal of sprays (41). Marth, Batjer, and Moon (28) have also compared dusts with sprays, using Stayman Winesap as the test variety, and reported comparable results. The uncertainty

of adequate coverage inherent in the dusting operation probably accounts for its minor usage in commercial hormone control of fruit drop.

Tukey and Hamner (49) and also Marth *et al.* (28), using a highly concentrated solution of hormone in hand aerosol bombs, found this method of application to be effective. Obviously its usefulness is restricted to small trees, although Hamner and Rasmussen (18) found that a concentrated oil solution of NAA applied as a vapor by a commercial fog machine (Todd Insecticidal Fog Applicator) could be used successfully on standard-size trees. The uncontrolled drift of the fog with even slight air currents is the chief limiting factor.

Since the first trials in 1944 the apple and pear growers of the Pacific Northwest have made increasing use of airplane applications. Naphthaleneacetic acid dissolved in an oil emulsion in high concentration is applied by low-flying planes equipped to disperse the material in minute droplets which are forced down through the trees by air turbulence created by the planes. This method of application has obvious advantages in covering large acreages quickly and, according to tests conducted by Thompson and Batjer (48), it is quite effective in fruit drop control, although apparently not the equivalent of a thorough, conventional spray application.

There is no question but that thorough coverage with the applied growth substance is important for maximum drop control regardless of the method of application. While there is some transmission effect through the tissues for short distances, at least in the case of NAA, such effects are quite limited. Batjer and Thompson (5), carefully applying this compound by hand to fruit stems and cluster bases and to the subtending foliage only, found that this foliage was the chief means of transmitting the effect to the point of fruit abscission. There was no evidence of transmission, however, from a completely sprayed spur to unsprayed spurs nearby on the same branch. It is entirely possible that in the case of 2,4-D the transmission effects take place over much greater distances in the case of the few varieties on which it is effective. The ability to affect abscission by transmission over considerable distance within the plant tissues will be an important characteristic of new compounds destined for fruit drop control.

Varietal differences.—While all apple varieties are apparently affected by NAA and its amide, the response varies greatly depending on the

variety. In general the hormone sprays are more effective on early-maturing types than on late varieties, but there are exceptions to this generality. It is probable that in early varieties not only are tissues of the stem and also the leaves in a more reactive condition, but also that the sprays are applied during periods of higher temperatures than in the case of late varieties.

Another factor which may account for the observed varietal differences in response of apples is the variation in the mode of the abscission process. McCown (30) stated that varieties differ in the order in which the various tissues in the abscission zone begin their process of abscission and that those varieties in which the pith abscission is delayed until after the bark tissues show signs of separation respond most readily to hormone sprays. In the case of McIntosh, a variety on which hormone sprays are generally reported as being effective for a period of only 10 days, he pointed out that abscission in the pith tissues begins very early, by the time there is noticeable striping of the fruit, and he questioned whether hormones could be very effective after this time. This observation may also explain the general lack of appreciably longer response of McIntosh to a second spraying, as has been reported by Batjer and Marth (2), Murphy (34), and others, although with some varieties this procedure is effective.

The existence of varietal differences in apples in their response to 2,4-D has already been noted, most varieties being quite indifferent to this compound in both tree and fruit reactions. Edgerton (13) has published some results comparing the effect of NAA and its methyl ester and also 2,4-D and its methyl and amyl esters on apple petiole drop and fruit drop in three varieties. The petiole drop test consists of cutting uniform leafy shoots from the trees and placing their bases in water, clipping off the leaf blades, spraying the petioles with the growth-substance solutions, and recording subsequent petiole abscission over a period of days. Edgerton found that the petioles of Stayman Winesap and Winesap varieties responded to 2,4-D and its esters, thus agreeing with the observed response in fruit drop control with these two varieties. McIntosh, on the other hand, failed to respond to these compounds in both fruit and petiole abscission. On Stayman Winesap and Winesap the order of effectiveness of the various compounds on petiole drop agreed quite well with the observed order of effectiveness in control of fruit drop. It would appear from the limited evidence thus far

available that the petiole-drop test may be a simple and useful means not only of screening varieties for response to a particular compound but also of screening new compounds for effectiveness in controlling drop of a particular kind of fruit.

Temperature.—It is recognized that hormone responses in general are favored by high temperatures and impeded by cold. Without any precise information available, it was thought that temperatures lower than 70°F. at the time of spraying were unfavorable for drop control even though a temperature rise took place later on. In this connection the work of Batjer (1) is of considerable interest. He found that NAA sprays applied at midday with the temperature at approximately 80°F. were consistently more effective than sprays applied in the early morning of the same day with the temperature approximately 20 degrees lower. From a practical standpoint the difference in control was not great, and there was some indication that the unfavorable influence of the lower temperatures might be compensated for by using a higher concentration of the hormone. The work of Overholser *et al.* (35) is in complete agreement on the point of temperature effects at time of spraying. Batjer, in attempting to study the temperature relationship more closely, used the apple petiole technique in which the leaf blades were removed and the petioles sprayed at a controlled range of temperatures. The subsequent rate of abscission of the petioles was recorded at a uniform temperature for all lots. The results indicated that above 72°F. temperature effects were negligible. Below 72°F. the control of abscission was increasingly poorer with lowering of the temperature.

Fruit Thinning and Fruit Set

As all fruitgrowers know, thinning by hand is a tedious and expensive operation. Thinning is frequently necessary, however, in order to secure fruit of marketable size and to insure regular annual bearing with certain varieties that are prone to overcrop one year and fail to set the next. Numerous studies have been made to establish a practical thinning procedure by use of certain caustic sprays. The results have been variable because the process is dependent on killing or injuring only a portion of the flowers and young fruits, causing them to abscise. The procedure is not without hazard since the margin of safety is narrow before injury to the foliage and twigs occurs. Early thinning is also complicated by the risk that frost may subsequently kill additional flowers or fruit and thus

the crop is overthinned. Hormone sprays, while not a panacea for all of these hazards, do offer the possibility of thinning without injury to the young foliage since their action is not necessarily a caustic one.

A discussion of the role of growth substances in fruit thinning must necessarily consider their effect on fruit set since one process is the opposite of the other, and the concept of thinning by means of these compounds was the outgrowth of the unsuccessful attempts with tree fruits to increase fruit set. For the purpose of this discussion, fruit set is considered to be the net resulting crop after all abscission, from flowering through the June drop has occurred.

The writer is not aware of any well-substantiated case in tree fruits of an improvement in fruit set by means of growth substances. This statement does not apply to the few cases of those fruits that can be stimulated to set parthenocarpically and where an improved set is the result of such stimulation coupled perhaps with faulty or complete lack of pollination. On the other hand, there are many recorded failures of the hormone chemicals to improve set and instances in which fruit set was actually decreased.

The success with growth substances in controlling mature fruit drop naturally led to trials to increase crop production by preventing the abscission of young fruits. Gardner, Marth, and Batjer (17) recorded the failure of NAA and its amide, applied at petal fall, to improve the set of several apple varieties but made no mention of any resulting decrease in set. Burkholder and McCown (10) found that NAA sprays at 10 ppm. applied to Starking at full bloom reduced the number of clusters that held fruit past the June drop by 15.1 per cent, and at 50 ppm. the reduction was 77.7 per cent; the amide at 50 ppm. reduced the set by 34.0 per cent. Severe epinasty and leaf scorch accompanied the use of 50 ppm. of NAA but no injury attended the amide. If the thought of using either of these substances as intentional fruit thinners occurred to these workers at the time, it was not mentioned.

With certain citrus varieties one might logically expect an improvement in set with growth substances since many varieties are apparently able to set parthenocarpically even without the benefit of applied stimulation. Pomeroy and Aldrich (50), however, made extensive trials on Washington Navel oranges and Marsh grapefruit without success. They used several growth substances, both naphthalene and indole compounds, at various concentrations and applied in different ways. Naphthalene-

acetic acid at relatively high concentrations reduced the set rather than increased it. Stewart and Heild (44) using 2,4-D also noted a reduction in number of orange fruits set, although not necessarily a reduction in number of harvested boxes because the fruits remaining grew to larger size. The lack of response of citrus varieties in setting additional fruit under hormone stimulation is especially puzzling in view of its naturally parthenocarpic tendency.

Failure of the hormones to prevent the abscission of young fruits is not restricted to the fruits mentioned. Negative results are frequently deemed of such little interest that they are never published. In Florida the Haden mango blooms profusely but sheds its small fruits to the degree that satisfactory crops are infrequent. The June drop of avocados often converts a seemingly heavy set to a very light crop. Gardner and others in Florida (unpublished results) used various hormone chemicals on these two fruits without benefit. Smith (38), in the hope of controlling the immature drop of pecans, found no effect on shedding from applications of indoleacetic acid or naphthaleneacetamide at 50 ppm. Naphthaleneacetic acid at this concentration, however, gave a definite increase in the shedding of young nuts.

If one were to generalize from the above record it would be concluded that with tree fruits hormone treatments have either been without effect on fruit set or, in the higher concentrations, have resulted in reduced set. Schneider and Enzie (36,37) were apparently the first to utilize the abscission-promoting effect of these compounds on young fruits with the specific intent of fruit thinning. They reported that NAA sprays at 100 ppm. on apples nearly eliminated the crop on all varieties tested, but with marked injury to leaves and growing points. At 10 ppm. the effect was more moderate on both scores. Naphthaleneacetamide at 80 ppm. performed well in thinning without damage to foliage. Indole derivatives, however, were of no value. Hoffman, Southwick, and Edgerton (25,39) and also Batjer and Thompson (7) reported on the use of the sodium salt of NAA for thinning apples and in general found it a promising material although subject to variable results—overthinning in some instances and underthinning in others. This may be an expression of the variable state of the trees or of the conditions of spraying rather than an inherent shortcoming of the compound itself. It would appear that more attention should be given to naphthaleneacetamide as a thinning spray rather than to the acid or the sodium salt despite the

higher concentration of amide required. The absence of any deforming epinastic effects from the amide recommends its use where tender new growth is concerned. In the meantime, the apple industry is making increased use of NAA and its sodium salt for apple thinning and will probably continue to do so until more effective and more consistent materials are found.

In the case of peaches it is of interest to note that Southwick *et al.* (40) found no thinning effect from either the sodium salt of NAA up to 40 ppm. or its methyl ester up to 20 ppm. in the sprays. Murneek and Hibbard (33) also reported the use on peaches of the sodium salt as a thinning spray in the range of 5 to 40 ppm., but the conditions of their experiment were such as to make interpretation of the results somewhat difficult. In one test there appeared to be an actual increase in set over the controls, but the counts were made before drop was completed. The data of another test appeared to show a slight reduction in set with the use of hormone sprays although, without the benefit of any statistical evaluation, it is questionable that the difference is significant. It should be recalled that applications of the naphthalene derivatives have also been shown to be without effect on the preharvest drop of peaches.

Growth Substance Effect in Relation to Stage of Development

From the previous discussion it would appear that the available hormone chemicals offer a possible solution to problems involving a delay of the preharvest abscission, whereas in the case of flower and young fruit abscission the same compounds offer no hope of increasing fruit set and in fact, in many instances, will promote fruit shedding. The reason for this seemingly different action of growth substances at these different stages of development is of fundamental importance in the approach to any problem involving abscission. Admittedly the question calls for more investigation, but with little information available some speculation may be permissible.

Type of abscission.—In studying the abscission of apple fruits both MacDaniels (26) and McCown (30) found that the anatomical changes accompanying the preharvest drop differ in character from those of the early drop of young fruits and flowers. Early drop according to these workers is associated with a definite preformed abscission layer resulting from secondary cell division. In late drop the changes are characterized

chiefly by alterations in the cell walls in an abscission zone. It must be assumed that the action of the hormones in the late drop is to delay the weakening of the cell-wall material so that separation of the cells is slowed, but that these compounds do not prevent the secondary cell division which results in the formation of an abscission layer observed in the case of early drop. The fact should not be overlooked that growth substances may also delay apple leaf abscission, particularly in instances where the leaf blades have been removed as in the petiole test for hormone activity. Delay of leaf fall in apple orchards has also been observed in cases where 2,4-D was used. No description has been found in the literature of the mode of abscission of apple leaves, but in certain other plants the process appears to be a combination of cell-wall changes in certain tissues and abscission layer formation in other tissues.

Both early and late abscission delayed.—The possibility exists that there is actually no fundamental difference in the response of the tissues to growth substances in early and late abscission. Persistence of flowers, or at least of their petals, for an appreciable period following a hormone spray is a common observation. The usual occurrence following the spraying of young fruits, particularly with a rather high concentration, is a noticeable delay in their abscission, even though the subsequent drop is increased. Thus growth substances may have a direct effect in delaying the weakening of the cell walls regardless of the type of abscission involved. The increased drop of young fruits when abscission is resumed might be explained on the basis of competitive antagonism between the applied compounds and the natural auxins produced by the developing fruit which provides a steady supply to prevent abscission just as the leaf blade provides its petiole with an anti-abscission auxin. This is highly speculative but not without some basis of experimental evidence.

Problems Needing Solution

To date the record of the research worker in solving many of the fruitgrowers' problems by means of growth substances is not outstanding in relation to the seeming possibilities. It is true that control of pre-harvest drop has been rather thoroughly studied and the fruit industry has adopted these sprays as a regular practice and to great advantage. The use of hormone sprays for fruit thinning, an unexpected outgrowth from the attempts to improve set, is also gaining in orchard use. There

are, however, a number of important problems that would appear to be amenable to application of growth substances, on which little or no progress has been made. Some of these should be mentioned here.

Parthenocarpy.—The subject of parthenocarpy is ably reviewed in a separate paper in this symposium, and it is not the intent to encroach on that discussion here other than to point out that for our most important tree fruits, such as apples, peaches, pears, and the citrus varieties, no practical progress has been made in stimulating parthenocarpy. The work of Crane and Blondeau (9,11) with the Calimyrna fig may constitute a partial exception to this general statement. While most figs are naturally parthenocarpic, this variety is almost completely non-parthenocarpic in the sense that it does not hold its fruit to maturity unless pollinated, although it should be noted that considerable development of the syconia takes place without pollination. In the work of these investigators sprays of indolebutyric acid at 1,500 ppm. (9) and, of more practical significance, 2,4,5-trichlorophenoxyacetic acid at concentrations as low as 10 ppm. (11) caused the fruit to complete its development and at a greatly accelerated rate. The need for more work on parthenocarpic stimulation in tree fruits is real, in view of the number of self-sterile varieties and frequent unfavorable pollination conditions which result in poor set. There is also the possibility that by proper stimulation parthenocarpic fruit might be superior in some respects to fertilized fruit. The problem is intimately tied in with the general problem of improving fruit set. It should be pointed out, however, that the parthenocarpic response is a different reaction from simply the prevention of abscission, for it is possible to hold flowers and young fruits on the tree for appreciable periods, but without stimulation of ovary development.

Delay in flowering.—The greatest hazard in most sections in the production of many deciduous fruits, particularly those that tend to bloom early in the spring, is freezing temperature at flowering time. A delay of even 10 days in blossoming might save many crops from freezing damage. The inhibiting effect of many of the hormone chemicals on lateral leaf bud development is well known. Unfortunately flower buds are not as subject to inhibition by these compounds as are leaf buds. Winklepeck (51) applied a spray of NAA at 125 ppm. to peach trees just as the flower buds were swelling and beginning to break and reported that the sprayed trees arrived at full bloom two weeks later than the

controls. No mention was made of injury from the sprays. Mitchell and Cullinan (31) failed to confirm these promising results with peaches by using sprays applied in the spring. While the leaf buds were retarded the flower buds were either injured by the high concentrations of the growth substances or were not delayed in opening. Hitchcock and Zimmerman, working with peaches and several other fruits, reported (22) that a more effective time to apply the growth substances is during the preceding summer when differentiation of the flower buds takes place. Their report was most encouraging and did not emphasize any injurious effects from the high concentrations of the potassium salt of NAA used at this time of year. From subsequent work on peaches Marth, Havis, and Batjer (29), using the same compound at the same concentration range and same time of year, concluded that injury to buds and even to branches is so severe as to preclude use of this compound as a possible orchard treatment. Moreover, the delay in flowering was, at the best, only two days; and according to these workers, it was probably associated with injury rather than an inhibiting effect of the sprays. Usually the critical test of the feasibility of any suggested orchard practice is whether or not fruitgrowers adopt it. Growth substance applications for delay of flowering in fruit trees have not reached that stage, but certainly the importance of the problem warrants more investigation.

Breaking of dormancy.—In determining the southern limits of deciduous fruit culture probably no factor is more important than the chilling requirements necessary to terminate the rest period of the trees. During mild winters at the present southern limits of these fruits the chilling requirements may not be satisfied, with the result that the trees and their potential crops suffer from delayed foliation. It is not unlikely that dormancy involves a hormone relationship. The work of Bennett (8) points to a high auxin content in buds and shoots of pear trees as the dormant condition is entered, and a progressive disappearance of the auxin as the rest period is gradually broken. The growth-inhibiting effect of applied synthetic hormones on vegetative buds, particularly the effect of these compounds in maintaining dormancy of tubers, further supports a relationship between hormones and rest period. Obviously here is a problem in which it would be desirable to accomplish the opposite of prolonging dormancy. It is possible that to accomplish this end compounds may be necessary that have quite a different physio-

logical action from what we now think of as hormone activity. It is scarcely necessary to point out how important it would be to have a ready control over dormancy, being able both to impose and terminate it, and how such control could extend southward the culture of many deciduous trees.

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Use of Growth Substances in Tropical Agriculture

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S YNTHETIC plant hormones have found extensive use in tropical agri-
culture. From the point of view of progress in research, however,
the accent has been far too much on use and not enough on the under-
lying fundamental principles. In the long run a balance between theo-
retical and practical knowledge must exist. It is true that at times trial
and error methods can yield spectacular results. Yet, sooner or later
"bugs" develop which can be corrected only after theoretical knowledge
has caught up and the principles involved are more fully understood.

Tropical crops and plants make highly worthwhile subjects for studies
in fundamental plant physiology. Often physiological principles are
apparent in tropical plants while they are obscure in plants growing
under the different environment of the middle latitudes. It is no wonder,
therefore, that new principles are often brought to light by those working
in the tropics. Fitting, working in the tropics on orchids, was first to
realize the existence of hormone-like substances in the development of
the ovary, and, as a result of these observations, to use the word hormone
for the first time in plant physiology (57). It was Bouillenne and Went
(5), again working in the tropics on tropical plants, who conceived the
idea that substances now known as auxins promote the initiation of
adventitious roots on cuttings.

The enormous potentialities of plant physiological research in the
tropics is not sufficiently realized by those responsible for investing in
research. If money is invested in tropical research it is usually done with
immediate material benefits in view. However understandable this is,
there is a great need for the establishment of permanent institutions in
the tropics where plants are studied for the sole purpose of "extending
the horizons of our intellect" as Bronk (6) has so aptly put it.

In discussing the tropical uses of plant hormones, I will attempt to do so by including the underlying principles as far as they are known at present. They could be classified under the following headings: Fruiting, Rooting, and Weeding.

FRUITING

Growth-regulating substances are known to affect tropical fruit crops in a variety of ways. They may promote the flower initiation of the plant. This can be done directly, as in the pineapple, or indirectly, as in litchi. The synthetic hormones may also regulate the fruit growth and development after the flowers have been initiated naturally, as is exemplified by the fig. Finally, after the fruit has been formed by natural processes, hormones may delay its abscission from the tree and improve its keeping qualities. This is illustrated by the orange. Each of these effects will be dealt with below.

Crop control in the pineapple.—The pineapple plant produces in its lifetime only one fruit. Flowering, under natural conditions, starts in the fall and continues through the winter (Fig. 2). Approximately six months after flower formation the fruit is ready for harvest. The age at which a pineapple plant begins to flower depends upon its variety and the external conditions. On the average, when a pineapple plant during the flowering season reaches an age of 18 months it is capable of producing a marketable fruit. Some varieties like the Puerto Rican Cabezona take occasionally as long as 5 years before they flower. Such slow varieties can be brought into earlier production by treatment with synthetic plant hormones (48). These hormones will also cause flowering in very young plants too immature to flower in the natural season.

In the tropics the growing of plants is not restricted by frost and similar factors which so drastically curtail crop production in our middle latitudes. There is no reason, therefore, why a crop like the pineapple cannot be made to yield beyond its natural season, or even throughout the entire year. In the pineapple industry several methods have been in use for several decades by which growers have succeeded in extending the harvest season.

It began when it was accidentally discovered in the Azores that smoke will force the plants into early flowering (46). Older growers in Puerto Rico still remember how tents were erected over the rows under which a smoky fire was made during the night. An investigation of the active

Figure 1. The application of synthetic plant hormones in a Hawaiian pineapple plantation. The hormones force all plants uniformly into flower, thus extending the harvest season and making picking operations more efficient.

components of the smoke showed that its effect is due to unsaturated hydrocarbons, principally ethylene (35). Acetylene also was found to be active. These unsaturated hydrocarbons found a widespread use in the pineapple industry. At present their use has declined considerably, as they are being replaced by synthetic plant hormones.

In 1939 it was discovered that naphthaleneacetic acid (NAA)* could force pineapple plants into flower (10). This compound has been accepted by many growers as the best of the flower-inducing agents which are
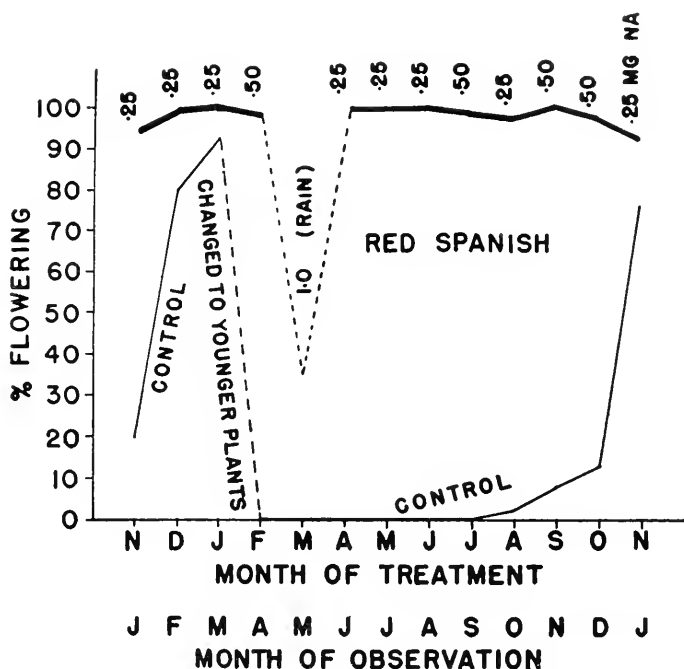
Figure 2. Experiment showing flower induction in pineapples of the Red Spanish variety in Puerto Rico. Each month 125 plants were treated with 5 different concentrations of naphthaleneacetic acid (NAA), 5 cc. of which was poured in the center of the plant. The amount of NAA per plant which gave maximal flower induction is shown above each point of the curve; one treatment was washed out by rain. The course of normal flowering is indicated by the control curve. The results show that by the use of NAA nearly 100 per cent flowering can be obtained throughout the entire year.

*Editor's Note: In this paper as originally submitted, the abbreviations for naphthaleneacetic acid and indoleacetic acid were NA and IA. For consistency, these have been changed to NAA and IAA, respectively.

available at present. In Hawaii especially, NAA is used extensively. Under current commercial practice approximately 25 grams of the sodium salt are applied per acre. This is sprayed on by large ground equipment using spray booms 50 feet long (Fig. 1). The cost of the chemical is only about fifty cents an acre at present, while the total cost of treatment is roughly five dollars an acre. Only one treatment is required for flower induction.

The advantages of forced flower induction in the pineapple industry do not lie exclusively in an extension of the harvest season. The much improved uniformity and regularity of flowering and consequently of fruit production is of equal importance. Prior to the introduction of the practice of forcing by chemical means, the earliest and the latest fruits produced in a field during one season might be months apart. This entailed repeated harvests for one and the same field. At present, with chemical flower induction, the entire field is forced into flower at once, and therefore all fruits are ripe at the same time. Thus the entire crop of one specific field can be harvested with a single operation. Still another advantage of the use of chemical flower-inducing agents is that they increase the yield of the fruit per acre, as a larger percentage of the plants is forced into flower than without treatment.

Systematic hormone treatments have now made planned harvesting a reality. These treatments are so made that when the harvesting in one field has been completed the crew with its trucks and other machinery moves on to the next. The frantic rush, so characteristic of most perishable fruit crops, has thereby largely been eliminated.

Chemical flower induction not only makes it possible to determine the date of the harvest, but also to estimate with reasonable accuracy the tonnage a field will produce. A glance at Figure 3 shows that the more leaves a plant has the larger the fruit it will produce. Since after flower formation has taken place the number of leaves on the plant does not further increase, it is possible, by the use of graphs such as given in Figure 3, to predict at the time flower-inducing treatments are made the average weight of the fruit that will be produced. Even though much remains to be desired one may say that for the pineapple, crop control is more complete than for any other fruit crop.

Flower induction with NAA has certain drawbacks. Part of these have already been overcome. Thus, the compound causes a peduncle which is more slender than usual. This may result in a loss of fruit due

to falling over. The application of β-naphthoxyacetic acid, applied after the fruit development is well under way, has corrected the difficulty (28). Another effect of the use of synthetic hormones applied in the course of fruit development is a delay in fruit maturation, causing an increase in fruit weight (11,28). This double hormone treatment, one for flower initiation and a later one for strengthening the peduncle and increasing
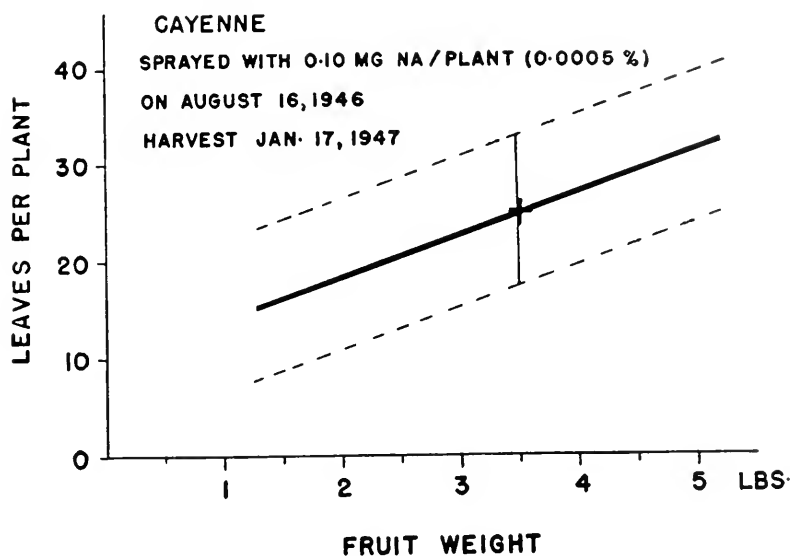
Figure 3. Regression curve showing the relation between the number of leaves of a pineapple plant and the weight of the fruit it produces. The data were obtained on 100 plants of the Smooth Cayenne variety growing on Vieques Island (P.R.) which were forced into flower by an aqueous spray of 0.0005 per cent NAA. The average number of leaves was 25.0 ± 0.53; and the average fruit weight 3.5 ± 0.07 lbs. The broken lines indicate the standard error of estimate.

the fruit weight, has been adopted as a standard practice in several of the large plantations.

A drawback which has not yet been overcome is that the use of NAA on pineapples of the Cayenne (Hawaiian) variety causes a reduction in the number of slips which the plant produces. These slips are branches which develop from lateral buds located on the upper part of the peduncle just under the fruit. They are important as replanting material. Not all pineapple varieties, however, suffer from a severely reduced

slip production as a result of NAA treatment. It is entirely possible that among the auxins a compound, or combination of compounds, may be found which does cause flower initiation, and yet does not suppress the lateral bud development.

Crop control in the pineapple is not complete without a means whereby it is possible to prevent precocious flowering. This has been accomplished by the application of large quantities of auxins. Theoretically this is understandable as it seems to be a general rule that auxins stimulate at low concentrations, while they inhibit these same reactions at high concentrations (27). From a practical standpoint this principle has not worked out satisfactorily for the control of undesired flowering in the pineapple. It is not impossible that growth inhibitors such as maleic hydrazide (36) might supply the solution to this problem.

As a result of the rapid progress in chemical growth regulation our fundamental concepts of which characteristics in a plant are desirable and which are not must undergo considerable change. Prior to chemical growth regulation it was desirable for a plant to flower readily during the natural flowering season, as this insured a high average annual yield per acre. At present, with cheap and efficient chemical growth regulation at our disposal, we would prefer plants which would not flower at all, except after chemical treatment. This would do away with the problem of precocious flowering, making planting throughout the entire year possible, thereby permitting fruit production on a commercial scale throughout the entire year. The Cabezona variety of Puerto Rico approaches these qualifications. It produces flowers readily after treatment with minute quantities of synthetic auxins, yet does not flower abundantly in the natural season (48).

Physiology of flower formation in the pineapple.—The pineapple is the only plant in which the application of known compounds will cause flower formation. Since, with the exception of ethylene and acetylene, these compounds belong to the group of the auxins, a study of the physiology of flowering is of more than local interest. Auxins are not species specific, and experience has taught that when an auxin elicits a certain reaction in one plant species it is likely to do likewise in most other species. Thus, when auxin causes pineapples to flower, it is most likely that in other plant species also it is involved in the process of flowering.

What do we know about the physiology of flower induction in the pineapple? In the first place we know that as minute amounts as 50 micrograms of synthetic auxins per plant will cause the vegetative growing point of the pineapple to change promptly into a floral apex (48). We also know that auxin exists inside the pineapple plant, and that the highest concentrations of native free auxin are found in the growing point (54). This auxin has been identified as indoleacetic acid (22). The immediate precursor of this indoleacetic acid in the pineapple plant is indoleacetaldehyde (23), which is stored in considerable quantities in the bases of the youngest leaves (54). Since these leaf bases are located in close proximity to the apex, it is likely that free auxin of the growing point originates from the precursor in the leaf bases of the youngest leaves. It is also known that the pineapple plant contains enzyme systems which convert tryptophan into indoleacetaldehyde and indole-acetic acid. Furthermore from the pineapple leaf an enzyme system has been isolated which inactivates indoleacetic acid (23). It is clear, there-fore, that the pineapple possesses an active auxin metabolism. This is again manifested by the curious geotropic flower induction of the Cabezona variety. When the vegetative plant is put on its side, it will not only right itself by a normal geotropic process, but in addition it will go into the flowering stage completely out of season and without the benefit of treatment with chemicals. The phenomenon has been in-terpreted as being controlled by the native auxin (50).

Under natural conditions flowering is brought about by a drop in night temperature during the winter (51). Photoperiodic effects seem of minor consequence in the pineapple. At present experimental data are lacking which link temperature and auxin induced flower induction. It has been suggested that this link may be found in the plant's organic acid metabolism (51). Also lacking is a link between these two types of flower induction and the induction by unsaturated hydrocarbons. No evidence was found that ethylene treatment increases the auxin level of the plant; Cooper (12) found no change, while Carl Leopold (personal communication) found a slight but consistent decrease in the free auxin level.

Conclusions on the physiology of flower formation in the pineapple.— From the evidence available one is confronted with the facts that, on the one hand, an active auxin mechanism exists in the plant and that auxin

will bring about flower initiation. Yet, on the other hand, an increase in the auxin level per se does not seem to be necessary for flower formation in the pineapple.

One might therefore conclude that it is conceivable that ethylene treatment increases the response of the tissues to auxin, thereby bringing about an increase in the physiological activity of the auxin in the plant. This assumption does not seem unlikely when viewed in the light of a recent discovery on the role of auxin in cambial growth. In the January, 1949, issue of the *Vakblad voor Biologen* C. Reinders-Gouwentak (34) discloses that when auxin is applied to dormant branches of *Fraxinus* growth of the cambium is promoted along the entire length of the branch, provided, however, that this branch has been treated previously by ethylenechlorohydrin. Auxin by itself had only a slight effect on cambium growth in the immediate vicinity of a cut surface, while ethylenechlorohydrin by itself was entirely ineffective in this respect. Apparently the ethylenechlorohydrin changed the metabolism of the branch, making it highly responsive to auxin. Similarly, ethylene might bring about metabolic changes in the vegetative apex of the pineapple, making the tissue more responsive to the auxin it contains, thereby bringing about flower formation.

Indirect flower induction in litchi.—In the Hawaiian Islands litchi trees flower and fruit so irregularly that it is often believed that this crop has few economic possibilities. It was found, however, that in litchi too, flowering may be induced by treatment with growth-regulating chemicals. In contrast to the pineapple where auxins promote flower formation directly, in the litchi flower formation is due to the suppression of lush young shoots by auxins. A concentration of 50 ppm. of NAA applied before October immediately stops vegetative development (37). As a result of such treatments 88 per cent of the trees flowered, as compared to only 4 per cent in the controls. This indirect type of flower induction in litchi is probably due to a building up of nutrients and growth factors which normally would have been translocated away to the rapidly growing vegetative branches. This effect is comparable to flower induction by girdling of juvenile citrus trees (19), and of the rotenone-producing Peruvian *Lonchocarpus* (14). Here as well as in litchi trees flower induction apparently is also the result of the accumulation of nutrients and growth factors.

Shortening of the ripening period of the fig.—Although most commercial

fig varieties are parthenocarpic, the Calimyrna fig requires pollination by a specific wasp. It has been shown that when the mature pollen-receptive inflorescences are sprayed with suitable auxin preparations, the Calimyrna fig also can be forced into producing parthenocarpic fruit (2). This in itself might not be of great physiological interest were it not for the fact that some of the auxins reduce the normal ripening period of the fruit so drastically that the average 120 day period is cut in half. This was the first example where auxins cause a drastic reduction in ripening time of fruit; recently it has also been shown in apples and peaches.

The case assumes further interest if the active compounds are examined (3,16). 4-Chlorophenoxyacetic acid will cause ripening of the fruit without materially speeding up the ripening period. Except for the absence of seeds parthenocarpic fruit produced with this chemical resembles that produced by normal pollination. It is entirely possible that this treatment will replace the cumbersome caprification by the wasp. Quantities of approximately 100 grams of 4-chlorophenoxyacetic acid per acre, at 40 to 60 ppm., are required.

When an extra chlorine atom is added to the molecule so that 2,4-dichlorophenoxyacetic acid is formed, a compound of considerably lower activity than the original 4-chlorophenoxyacetic acid is obtained. The activity of this dichloro compound is so low that it was originally thought to be inactive. Recently it was found that it can bring about parthenocarpy in the fig in concentrations in excess of 250 ppm. (40). When still another chlorine atom is added to form 2,4,5-trichlorophenoxyacetic acid, a compound of unusual physiological activity is obtained. Not only does it cause parthenocarpic fruit setting at 10 ppm., but in addition it hastens the ripening period. As is shown in Figure 4, curve A, it does so by omitting the rest period between the initial and the final stage of rapid fruit growth. The trichloro compound also will accelerate ripening when applied to pollinated Calimyrna fruit (Fig. 4, curve B), and to naturally parthenocarpic fruit of varieties such as the Black Mission. Why such closely related chemicals have such widely different effects, which, however, are more quantitative than qualitative, is not known at present.

The double sigmoid growth curve C of Figure 4 reflects two periods of rapid fruit growth separated by a rest period. Many explanations have been offered for the occurrence of this rest period. In view of its successful

elimination by treatment with a suitable auxin, it would seem justified to conclude that a low auxin level is the causal agent (16).

Increased fruit ripening in the banana.—Speeding up of fruit ripening by auxins has also been observed in bananas after they have been removed from the plant; a thorough spraying with .02 to .05 per cent of 2,4-dichlorophenoxyacetic acid replaces the customary ethylene-induced
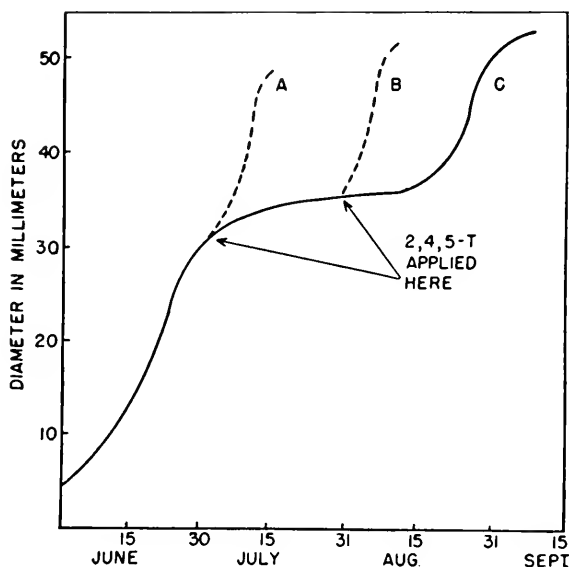
Figure 4. Experiment showing acceleration of fruit ripening of the Calimyrna fig by treatment with an aqueous spray containing 25 ppm. of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Normal fruit growth is shown by the solid curve. The first part of this curve is due to growth of the fruit (syconium) prior to pollination, which took place about June 30. Without pollination or suitable auxin treatment fruits drop off shortly after this time. Treatment at this time of the nonpollinated fruit causes growth represented by curve A. Pollinated fruit treated on July 31 immediately came out of the rest period and followed curve B. (3).

ripening (30). The effect may be attributed to the known stimulatory effect of auxins on the amylolytic enzyme system in plants (29).

Uses of auxins on citrus fruit.—Preharvest drop of citrus fruit is a serious problem, and when it was shown (20) that NAA can control a similar drop in apples, attempts were made to reduce drop in citrus by similar means. These attempts all met with failure until 2,4-D was

tried (41). This compound persists longer in the plant than NAA which may account for its effectiveness in citrus. Concentrations of about 8 ppm. of 2,4-D applied as a drenching aqueous spray to the trees have reduced preharvest drop in Valencia and Navel oranges (41), as well as in grapefruit (42).

Further beneficial effects of 2,4-D were discovered. Thus, it turned out that treated fruit after storage kept better than the nontreated controls because less black button developed. In addition the fruit stems of treated plants showed less die-back (43). The storage life of lemons also could be increased by 2,4-D treatments of the fruit before storage.

One of the difficulties often associated with insecticidal oil emulsion sprays on citrus has been an increased drop of leaves and immature fruit. It was now found that the addition of 2,4-D to these emulsions could check this drop. 2,4-D is now added to oil emulsions for a dual control program (43); not only does it check the fruit and leaf drop, but it reduces fruit stem die-back, and during storage cuts losses due to black button. An ester form of 2,4-D sprayed on at 4 ppm. in terms of the final emulsion is most efficient. Related chlorinated phenoxyacetic acids seem to have effects comparable to 2,4-D; 2,4,5-T may even be more effective (39).

ROOTING

After the discovery that auxins promote root formation on cuttings (45) general use has been made of this principle. One might say that auxins have been tried on almost every plant for which vegetative propagation might offer advantages. The results of all these trials, which include many tropical species, have been summarized in extensive tabular form (1,30,31,44). It is intended to discuss here first an example of the use of growth substances in the vegetative propagation of a tropical crop, to be followed by some fundamental aspects of the process of root formation as worked out on a tropical plant in the tropics.

Root formation on cacao cuttings.—The use of synthetic plant hormones for the promotion of root formation on cuttings as applied in the tropics can perhaps be illustrated best by its use in the vegetative propagation of cacao in Costa Rica. In order to appreciate the importance of cacao in the economy of Central American agriculture a few words must be said about the banana culture with which the cacao culture is closely allied. When the banana industry came into being in this area at the

beginning of the present century the plants grew well anywhere in the coastal lands. In 1910 a wilt was reported which started to invade plantations especially in the moist areas with acid soils. By 1920 this wilt, caused by *Fusarium oxysporum cubense*, had become a major problem which finally drove the industry from the original plantations (32). Thus, new jungle had to be cleared for new banana plantations and new crops had to be found to occupy the abandoned banana lands. Cacao proved to be one of several crops suitable for replanting the old banana lands. In order to make culture profitable it is necessary that the plantations consist of trees which combine a high yield of high quality fruit with a reasonable resistance against diseases. This is only practical at present by the vegetative propagation of parent plants of proven outstanding performance in these respects. In cacao this propagation is done most profitably by cuttings, which brings us back to our starting point.

When cuttings are taken from a cacao tree without any special precautions, and then planted in a propagator, they will wilt and fail to form roots. The reason for this is that the bark of the cacao tree contains mucoid material. When the cutting is taken from the branch this will ooze out and plug the xylem vessels, thus causing the death of the cutting by water starvation. When the cut stem ends are kept in water before being transferred to the propagator much of this trouble can be avoided. This is an example of how a seemingly minute detail may spell success or failure of a technical process.

There are many other conditions which have to be observed for the successful rooting of cacao cuttings (8,9,18). Without auxin treatment, however, root formation will still be unsatisfactory. When, however, cuttings are treated with a suitable auxin prior to being placed in the propagator, the following benefits result: roots are formed faster; a larger percentage of the cuttings forms roots in a shorter time; and the cuttings are more easily transplanted because under auxin treatment a compact root system composed of many short roots results. These are the reasons that synthetic plant hormones have become an integral part of propagation by means of cuttings.

The most widely used of the synthetic auxins for this purpose is indolebutyric acid (IB). It is used either alone or in combination with other auxins such as NAA (24). In practice it is most frequently formulated as a powder. The moistened ends of the cuttings are dipped into

Figure 5. Coffee cuttings showing the necessity of leaves for root formation. All cuttings were treated at the base by dipping into 50 per cent ethyl alcohol containing 2 mg. of indolebutyric acid (IB) per cc. They were taken from 1 year old plants and photographed 50 days later; the defoliated cuttings had regenerated some small leaves at that time. The experiments took place in Puerto Rico.

this powder before being placed in the propagator. The author has found the alcohol dip method (13) also highly practical. The cutting ends are dipped in 50 per cent alcohol containing a relatively high concentration of a suitable auxin, such as 2 mg. of IB per cc.

Recently attention has been called to the effectiveness of some of the chlorinated phenoxy acids (25). In view of the striking results that this class of synthetic auxins gave in the citrus and fig industry in the regulation of fruit production, it is not unlikely that they may find application in some cases where IB or NAA failed to induce roots.

Some physiological aspects of auxin action in cuttings.—Cuttings of many plant species have responded to auxin treatment, yet many remain that as yet have not produced roots. A number of woody species such as *Hevea*, coffee, and mango will form roots when the cuttings are taken from young parent plants but fail when they are taken from older trees. The reasons for failure to root are not well understood at present. It would seem that it is not due to lack of auxins but rather to lack of proper cofactors. Auxins will act only when cofactor requirements are satisfied, or in other words, when the tissue is in reactive condition. There are many examples of this in auxin physiology. Auxins will cause tumor formation only when the tissue has been prepared by *Bacillus tumefaciens* (see 47). Auxin will cause cambial growth only if the tissue has been made reactive to it; this can be done by treating dormant branches with ethylenechlorohydrin (34). In cuttings too, auxin will cause root formation only when the tissue has been put in a responsive condition. What this involves has been analyzed in *Hibiscus* cuttings (52) and will be briefly discussed.

It is known by horticulturists that the presence of leaves on cuttings greatly increases the chances of successful root formation (15). This effect of leaves is demonstrated on coffee cuttings in Figure 5. It will be seen that even though both the leafy and the defoliated cuttings received the same auxin (IB) treatment, only the leafy cuttings responded with root formation. This effect is further elaborated in Figure 6 for *Hibiscus* cuttings. In these the terminal bud was removed in order to avoid complications with the naturally produced auxin. Without leaves and without auxin (IB) treatment no roots were formed, as indicated by point A in Figure 6. Without auxin treatment an increase of the number of leaves left on the cuttings is also ineffective for root formation (curve AB). Defoliated cuttings treated at the base by dipping

them in 50 per cent alcohol containing 2 mg. of IB per cc. also failed to root (point C). However, when this auxin treatment was given to leafy cuttings, roots were formed in proportion to the number of leaves present on them (curve CD). Leaves, then, provide factors which together with auxin cause root formation. At present we have also learned something about the chemical nature of these factors.

In order to investigate which factors were contributed by the *Hibiscus* leaves to the cutting, defoliated *Hibiscus* cuttings were treated at the base with a number of compounds which conceivably might be auxin cofactors. This was followed by the usual IB treatment. One of the first compounds to be tried as a possible cofactor was sucrose. It has been known for years to stimulate root formation on cuttings (17), and was indeed found active in the defoliated, IB-treated *Hibiscus* cuttings (52). Yet the number of roots thus formed was still far below that formed
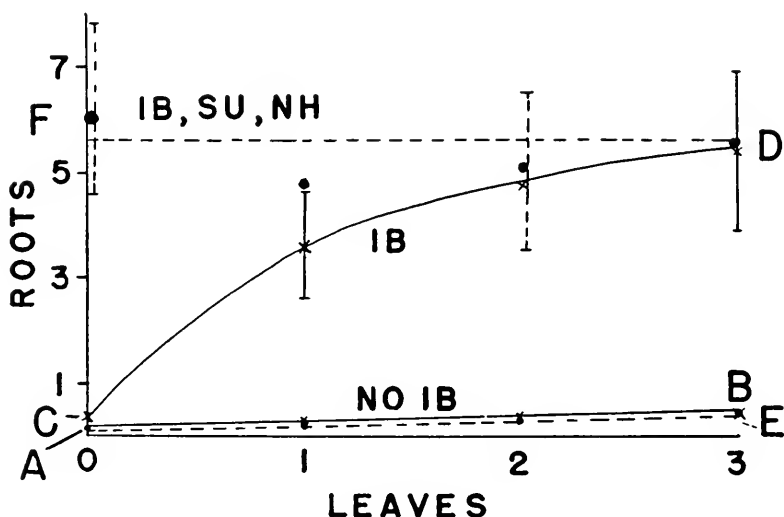
Figure 6. Graph showing how root formation on *Hibiscus* cuttings depends upon both auxin and the presence of leaves. AB shows that without auxin leaves are ineffective. CD shows that in auxin treated cuttings (IB treatments as in Figure 5) the number of roots formed increases with the number of leaves left on the cutting. F shows that the effect of leaves in auxin treated cuttings can be replaced by treatment at the base with 4 per cent sucrose and 0.1 per cent ammonium sulfate; sucrose and 10 ppm. arginine has a similar effect. AE (dotted line) shows that without auxin treatment sucrose and ammonium sulfate are ineffective, even in the presence of leaves. (52).

on leafy cuttings. Next it was found that ammonium sulfate, given together with sugar, would increase the number of roots of IB-treated defoliated cuttings so much that it was equal to that of IB-treated leafy cuttings. This is represented by point F in Figure 6. The same treatment given to leafy cuttings did not further increase the number of roots formed (curve FD).

Since it is unlikely that leaves would contribute ammonium sulfate to the cutting, a search was made for more likely substances. Among the most promising of these is arginine. When tested in the presence of 4 per cent sucrose 10 ppm. of arginine would produce an effect equivalent to 1,000 ppm. of ammonium sulfate (52). Since both arginine and sucrose are natural constituents of the plant it seems reasonable to assume that one of the functions of the leaf is to provide cuttings with these compounds, which then together with auxin cause root formation.

Arginine has recently also been recognized as a co-factor for auxin action in the elongation of the *Avena* coleoptile (4); it has a function as a high energy phosphate acceptor and accumulator. This would make its action as a cofactor to auxin understandable because auxin itself appears to be intimately connected with the phosphate metabolism of the plant, possibly with the transfer of high energy phosphate.

WEEDING

With respect to dollar volume of sales, 2,4-D for weed control undoubtedly constitutes the most important use of a synthetic plant hormone. In 1948 an estimated 16,000,000 pounds of 2,4-D compounds were manufactured and sold at an average price of $0.75 per pound (21). This compound is most frequently used as a selective herbicide on crops belonging to the family of the *Gramineae*. Consequently, it is finding its major use in the tropics in the culture of sugar cane (55). The aerial portions of sugar cane are practically completely resistant to 2,4-D sprays (49), but its roots are quite sensitive (7).

Against susceptible tropical sugar cane weeds such as *Commelina* and *Ipomea*, 2,4-D is highly effective. The small dosages necessary to kill these weeds make low-volume applications of 2 to 4 gallons per acre feasible. When the acreage involved is large enough such low-volume sprays can be best applied by plane. Most efficient of these sprays is 2,4-D in an ester form dissolved in oil, which, however, can be flown on safely only at times when there is a minimum danger of drift, and, as an

added precaution, when there are no susceptible crops near by. From the point of view of drift damage to susceptible crops dust formulations of 2,4-D are the most dangerous, and within the continental United States the Civil Aeronautics Commission will not grant licenses for 2,4-D dust applications. In the tropics dusts are still used. In areas with dusty soils there is considerable danger of 2,4-D drifting to adjacent areas even though the substance may be applied in an aqueous form. The finely pulverized soil serves as a vehicle which carries the absorbed 2,4-D to localities where it is not desired.

The exclusive use of 2,4-D as a selective herbicide brings with it the danger that the weed population which is effectively eradicated by it will be replaced by a group of plant species resistant to it (53). Thus it was observed in sugar-cane fields in Puerto Rico that the easy-to-kill *Commelina* was being replaced by the more resistant *Poinsettia heterophylla* and highly resistant grasses such as *Digitaria*.

2,4-D is used also as a so-called pre-emergence spray. In sugar-cane technology this term is used with reference to the emergence of the weeds, while in the middle latitudes it usually refers to the crop emergence. For pre-emergence purposes the 2,4-D is applied in such large dosages that it prevents the germination of weed seeds, including those of grasses. Amounts up to 5 pounds of 2,4-D per acre are applied. Generally speaking no damage is done to the sugar cane if this amount is not exceeded. Whether or not damage is done to the crop by pre-emergence applications of 2,4-D depends greatly upon a combination of soil properties and rainfall. These conditions are quite well understood at present due to the work done at the Hawaiian Sugar Planters Experiment Station (7).

The principle upon which pre-emergence weed control in sugar cane depends is that the 2,4-D stays in the surface layers of the soil. When this condition is realized the 2,4-D will prevent weed seeds from developing, while it will not interfere with the cane roots which are growing below the zone in which 2,4-D is present. The shoot of the cane which is relatively insensitive to 2,4-D will penetrate through the 2,4-D layer unharmed.

The Hawaiian investigators showed (7) that under the influence of rain 2,4-D moves downward through the soil as a concentration front comparable to those seen in chromatographic columns. In some soils this 2,4-D front moves very slightly even after much rain, while there

are other soils in which the 2,4-D concentration front moves downward to a considerable extent with only a slight amount of rain. Between these extremes a number of intermediate soil types exist. It is obvious that when conditions exist which will bring the 2,4-D front within the zone of growing sugar-cane roots, the latter are killed and the crop is lost. Such conditions may occur after heavy rains in soils which fix 2,4-D rather well; or again, they may occur after light rains in soils which hold 2,4-D only poorly. Ideal conditions for pre-emergence use of 2,4-D are those in which the herbicide remains fixed to the upper soil layers, thus preventing weed growth without interference with the deeper cane roots.

Relatively little definite knowledge is available on why 2,4-D is such an effective herbicide; neither do we know upon which properties its action as a selective herbicide rest. It is clear, however, that 2,4-D is an auxin and as such is translocated within the plant. It can reach the sensitive meristematic regions of the plant through normal channels because during the time of transport it is not toxic to the plant and for this reason does not interfere with its own translocation as is so often the case with some of the more violent plant poisons. After it arrives at the site of action, 2,4-D is not easily inactivated like the native auxin, indoleacetic acid. This persistence inside the plant is also in all probability a contributing factor to the high efficiency of 2,4-D as an herbicide. The depletion of carbohydrate reserves associated with the effect of 2,4-D on plants is not believed to be the primary cause of its phyto-toxicity (33,38). Interference with the aerobic respiration seems not unlikely as a primary cause. Germinating seeds (26) as well as bacteria (56) which require free oxygen for their respiration are smothered by 2,4-D, while those organisms capable of anaerobic respiration are not affected to any significant degree by it. For a more extensive discussion on tropical weed control the reader is referred to (52); for tropical weed species, to (58); and for the reasons for the physiological activity of 2,4-D as a herbicide, to (57).

Concluding Remarks

In conclusion one can say that the outstanding uses of synthetic plant hormones in the tropics are: the use of naphthaleneacetic acid in the pineapple industry, making crop regulation possible to an extent unequaled in other fruit crops; the use of indolebutyric acid in the whole-

sale propagation of cacao, making it possible to plant thousands of acres of young plants all with a proven record of high yield and quality coupled with disease resistance; the use of 2,4-dichlorophenoxyacetic acid as a weed killer in the sugar-cane industry, making it possible to eradicate weeds which could not be conquered by hoe and machete.

One may well ask what the future will bring. Here one has to distinguish between the immediate future and the years a generation or more beyond that. In the immediate future we may expect that an increasing number of uses will be found for plant hormones in the tropics. Tropical agriculture is expanding as an inevitable result of an increased world population. Plant hormone technology is also rapidly advancing. In addition, fundamental knowledge is slowly but surely increasing in the auxin field.

The outlook for the remote future seems less bright. Here no specific reference is made to plant hormones but to new applications of plant physiological research in general. These applications, so new that one cannot imagine them at present, depend invariably upon results obtained in fundamental research, that type of research which is undertaken with the sole aim of extending the horizons of our intellect. One could liken the relation between applied and fundamental research to the relation between logging operations and reforestation. The forests planted today will yield forest products tomorrow. On examining what fundamental research, of the type we are discussing, is being carried on today, we cannot be over-optimistic as far as plant physiology is concerned. In the tropics less fundamental research than ever is going on, even though technological research there is flourishing. In the middle latitudes, once-great European sources of knowledge have completely ceased to be productive. In our own country, despite a veritable boom in technological research, truly fundamental research in plant physiology is limited to a relatively few institutions. There is danger, indeed, that in our great desire for technological progress we are neglecting to nourish the source of it all, thereby slowly starving the goose that laid the golden eggs.

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Growth Substances
in Vegetative Development

The Role of Growth Substances in Vegetative
Development as Exemplified in Tissue Cultures

PHILIP R. WHITE

G ROWTH substances and their manipulation have played a considerable role in the development of plant tissue cultures as such, and in the control of their development and differentiation. Indeed Haberlandt's theory of wound hormones, leptohormones, and other growth substances arose originally out of his attempts to explain the difficulties encountered by himself and others in their attempts to establish plant tissue cultures.

Vegetative tissues of most if not all plants, when excised and placed on a moist substratum whether nutritive or not, will undergo a considerable initial enlargement due chiefly to hyperhydric increase in size of the surface cells. There is in general very little polarity to this enlargement if the fragment is small and if it is taken from a relatively undifferentiated region such as the pith, the cortical parenchyma, the interior of tubers or fleshy roots, etc. Larger or better differentiated masses may show some tendency to localized or even tubercular enlargement, but this is exceptional in the initial phases. This initial expansion is largely a physical response due to alteration in stresses, in the respiratory processes of the traumatized and exposed surfaces, in osmotic patterns, and so on. It has nothing whatever to do with normal proliferative growth. Indeed, tissues of some plants, such as those from mature tubers of *Helianthus tuberosa*, do not ordinarily show even this initial enlargement. These unresponsive tissues are now thought to be almost devoid of residual auxins, and the more usual initial enlargement is believed to be due, in part, to the activity of auxins which are present in most excised tissues. The ratio of available

auxin to exposed surface is supposed to account for the much more widespread and more pronounced enlargement obtained with relatively large explants.

Where present, this initial phase is commonly followed by the formation of an irregular cambium immediately below the surface, often involving cells which have undergone some enlargement. This cambium cuts off cells to the outside which are rapidly suberized. There is usually little or no new internal tissue formed. The cells of the interior of the fragment become woody, chlorophyll is formed in the outermost layers if the explants are exposed to light, and the fragment, although it may enlarge to as much as six or eight times its original volume, finally settles into a static condition in which it may remain alive and green but not growing for several months. If small fragments have been used enlargement is generally quite regular, the original outlines of the explant being recognizable after many months. Large fragments may give rise to localized, tubercular growths along with nongrowing or necrotic areas. If such a fragment is divided into two or four pieces and the parts are replanted on fresh substratum, the cut surfaces will undergo a similar series of changes but will again come to rest. The presence or absence of externally supplied nutritive substances, sugars, salts, and the like, apparently plays no role in this phase of development, which is entirely dependent, except for the moisture required, on residual supplies within the explant. It is sometimes possible to continue this subdivision through five or six passages, but it is not possible to establish continuously growing strains. This developmental pattern has been observed repeatedly since the days of Haberlandt and Rechinger. It is quite consistent and has led more than one early investigator to make premature claims to having established tissue cultures, before it was realized that a few such passages are not sufficient to establish the ability of such cultures to grow continuously. Neither Gautheret (1934—*Salix*) nor myself (1939—tomato and beet) has been free from this fault.

In 1939, however, Gautheret and Nobécourt independently showed that, if a growth substance such as indoleacetic acid or naphthaleneacetic acid is added at a suitable concentration to a complete nutrient either at the time of the initial excision or as a substratum for the first subcultures (that is to say after completion of the initial residual growth) when growth becomes dependent on external materials the

pattern can, in many cases, be radically changed. The degree and nature of the change depend on the concentration and activity of the growth substance used, as well as on the tissue involved. At low concentrations the tendency to initial hyperhydric enlargement is only slightly accentuated. The activity of the cambium, however, is greatly enhanced. Instead of being relatively superficial, the cambium tends to develop at a much greater depth in the fragment and to involve not a single layer but a considerable region. Instead of only a few superficial cork cells and deeper lignified masses, there is a proliferating region which is continuous both tangentially and radially, the new-formed cells tend to remain for a considerable time thin-walled, and there is formed an erratic but nevertheless clearly defined phloem on one side and xylem on the other. The initial arrangement of these layers may vary with the region from which the tissues were excised, but by gradual rearrangement the phloem always comes finally to occupy the superficial position. In addition the enlarged outer hyperhydric cells frequently undergo disoriented divisions resulting in the formation of nodular masses which, when they become sufficiently large, develop their own peripheral cambium and proceed as distinct centers of growth. Isolated areas of meristematic activity also often arise deep within the mass, especially in the neighborhood of necrotic areas. These localized centers, either superficial or deep-seated, give rise to the tuberculate and often loose and friable character of many rapidly growing plant tissue cultures. Subcultures can be made easily from such masses without important trauma. Indeed new growths frequently arise where a few cells have been accidentally dropped in manipulating the larger masses.

Two facts about these cultures appear at this stage of our ignorance to be of special importance. First, these cultures are still relatively disorganized, the only evidence of polarity being the radial polarity exemplified in the xylem-cambium-phloem orientation. In the second place the whole development of these cultures and the contrast between the static surviving masses discussed first and these rapidly growing, highly active disorganized masses has resulted from and is dependent on the presence of a certain amount of auxin in the substratum. Just how this auxin functions, aside from retarding differentiation (deposition of cell wall material) and enhancing mitosis (deposition of cytoplasmic proteins), is not at present clear even in a general way.

If the concentration of growth substances is increased somewhat more

the superficial hyperhydric response is further accentuated. The sub-surface cambial activity is initially not much changed, but instead of continuing unmodified for long periods these dividing regions are quickly organized into large numbers of well-defined root primordia. There are usually no stem growing points, although Nobécourt has reported the repeated, sporadic, and unexplained appearance of stem growing points and leaves on his carrot cultures. They are rare or entirely wanting in other laboratories and may indicate no more than a difference in varieties of carrot used. Such rooted cultures are, of course, quite useless as tissue cultures. They do, however, give us important leads in the study of auxin function. These cultures are not merely differentiated, but this differentiation possesses a definitely polar orientation. Moreover it is clear that here large numbers of roots have arisen without the presence of leaves or similar tissues from which rhizocalines in the classic sense could arise. The function of the hypo-thetical rhizocaline has been taken over, in dramatic manner, by either the heteroauxin itself or, secondarily, by substances arising in undif-ferentiated stem or root tissue under the influence of externally supplied auxin.

If, finally, the concentration of growth substance is still further in-creased, both polarity and organization are completely lost, and there remains only an exaggerated and pathological hyperhydricity. Enormous superficial cellular vesicles are formed, giving rise to a rapid and isodia-metric initial enlargement of the explant. These vesicles no longer appear to be capable of cell division so that the initial rapid mechanical enlarge-ment is followed shortly by necrosis and death. This behavior pattern at high auxin concentration is probably one of the facts responsible for the weed-killing action of such substances as 2,4-D, although excessive proliferation at lower auxin concentrations and, in the absence of in-creased nutrient supplies, subsequent nutritive exhaustion is undoubt-edly another major factor in weed killing.

These matters of cell size, cell number, and cell arrangement, of organization versus disorganization, of organization versus gross expan-sion, of continued function versus necrosis, and similar contrasting aspects of behavior can all be shown in tissue cultures to be under the control of growth substances. This is most clearly defined in the Jerusalem artichoke (*Helianthus tuberosa*) but is also demonstrable in varying degrees in tissue cultures of willow, hawthorn, grape, carrot, and many

other plants. The responses observed in these plants are cellular, generalized in character, and bear little apparent resemblance to the controls which auxins mediate in plants in nature, although these cellular activities are undoubtedly primarily responsible for the more specific reactions.

Much more specific responses can, however, be studied in tissue cultures of certain plants. If roots of dandelion, chicory, or related plants are cut up and the bits placed on moist sand or on a simple nutrient they will form buds on the upper surface and roots on the lower. This is true of almost any explant, no matter how small, if it will survive at all. Addition of auxin to the substratum in progressively increasing concentrations results in a progressive blocking of bud and root formation together with a considerable increase in the mass of callus formed on the surface. If auxin is applied locally to the upper surface of an explant in the form of an agar block, the blockage of bud formation and increase in callus development can be shown to center around the applied auxin and to become weaker with distance therefrom. The same result can in part be produced by implanting a preformed bud instead of using an agar block. The presence of the bud not only blocks the development of neighboring buds but also induces formation of a vascular strand which will connect the implant either with roots that may already be present or with newly formed roots. It does not, however, modify the amount of callus formed. This induction of vascular strands will take place through a cellophane sheet. Every indication is that it is mediated by some substance or substances of the general nature of the auxins.

This kind of approach has been extended by a double use of the tissue culture approach. Kulescha has shown by direct *Avena* tests that plant tissue cultures contain auxin in concentrations which are directly proportional to their rate of growth in culture and their degree of resemblance to tumors. Normal tissues which will not grow in culture without added auxin have little or none present. Tissues which have been habituated by prolonged cultivation on an auxin medium so that they are no longer dependent on external supplies show moderate levels of auxin content, while sterile crown gall tissues which grow in culture at a rapid rate are found to have a high auxin content. If, now, each type of tissue is grafted into the surface of slices of chicory, the inhibition of buds and the extent of callus formation induced parallel the observed auxin content of the implanted tissues. Normal tissue implants induce

little or no alteration in the callusing and budding behavior, while crown gall tissues completely suppress budding for some distance and bring about, per contra, an exuberant callus formation. Gautheret and Camus, and de Ropp, claim that the new callus itself possesses tumor qualities, but this should be re-examined.

From what I have said I think it is clear that the question of the role of auxins in the vegetative development of tissue cultures has not yet received the investigative attention that it deserves. Techniques for such studies do exist, however, and it is to be hoped that they will be utilized in the near future.

Factors Influencing the Growth of Plant Embryos

NANCY KENT ZIEBUR

THE growth of plant embryos has been the subject of many investigations during the last half century. It is a field of great theoretical interest particularly because of the opportunity that is provided for observing the development of adult structures from the cells that are initially the least differentiated. Two major problems have been considered in this connection. First, how is the embryo fed, which of the surrounding tissues contribute directly to its support, and what is the nature of the food material it receives? Second, to what extent is the pattern of embryonic growth autonomous, what is the role of the other seed parts in the control of embryonic development, and by what mechanisms is this control exerted? Solutions to these problems have been sought through both histological and physiological investigations, and by use of both natural and artificial conditions of growth.

A histological study by Nutman (8) deals with the evidence for the formation of growth-promoting substances in the developing rye kernel. He observed within the embryo sac and neighboring tissues a series of discontinuous growth phases associated with characteristic degeneration in certain parts of the developing fruit. Thus, soon after fertilization the synergids degenerate while simultaneously the antipodals divide; then when the antipodals degenerate, the endosperm nuclei begin to divide near the degenerating tissue, a characteristic nucellar strip undergoes a new development, and the embryo enlarges greatly. As the nucellar strip, in turn, is absorbed, the aleurone layer of the endosperm is formed. Finally, during the same general period a strict time association may be observed between the appearance of the stem, root, and

coleoptile primordia and the local degeneration of the endosperm in the regions of embryonic differentiation. The author postulates that hormones are liberated from the degenerating tissue, and that they influence nuclear divisions, cell enlargement, and tissue differentiation.

Among other studies of this general problem have been those of Brink and Cooper (2). Their approach has been to observe a variety of cases of arrested seed development, and to attempt from an analysis of these to arrive at an understanding of the important aspects of normal development. They conclude that in the angiosperms the endosperm typically plays an important part in the nutrition of the young embryo. The endosperm, because of its genetic constitution, seems well suited to compete successfully with the integuments which are at the start bigger than the embryo or endosperm, and which are themselves actively enlarging. The aggressive endosperm is able to establish itself as a physiologically dominant tissue in the seed and simultaneously to perform for the young embryo certain nutritional functions of which the embryo is not yet capable.

The endosperm is not, however, always of prime importance, as shown by Cooper and Brink (3) in their study of the common dandelion. This plant is an autonomous apomict, in which no fertilization occurs, either of the egg or of the central nucleus. In contrast to a related sexual species with the typically precocious endosperm, there appears to be with this material no regular interdependence of the embryo and endosperm. Many of the seeds show embryos that are much more advanced than the corresponding endosperms. One regular feature in the common dandelion is, however, the presence in the ovule, at the time the embryo starts developing, of an extensive amount of stored food material.

These examples are but a few of many studies which have shown that the very young embryo is not independent but must rely for its nutrition on the presence of some other tissue, be it endosperm or a functional substitute for it. The actual compositions of the materials used by the embryo have not been determined, but indirect evidence points to the importance both of hormones and of substances of direct nutritional value.

The control of the growth of older embryos, especially in the stages just prior to maturation, is also a question of interest. During the later portion of embryonic growth, embryos are generally autotrophic with respect to any special growth substances. They are often capable of

immediate germination, as shown by experiments with excised embryos *in vitro* and by germination tests on unripe seeds. The interest in the tissues surrounding the embryo now centers on the question of why an embryo able to germinate does not do so, and why instead it continues to grow embryonically until it is fully mature. This question is considered in a recent review paper by Evanari (5). A number of factors have been found which may prevent premature germination. These include high osmotic pressure, unfavorable pH, and a variety of chemical substances such as ethylene, organic acids, unsaturated lactones, aldehydes, essential oils, and alkaloids. They occur in all parts of plants, including the seed coat, endosperm, and the embryo itself. Their effects are mostly non-specific, although there are variations in the strength of the inhibition response and in the subsequent reaction of the posttreatment seedling.

The second approach to the whole problem of plant embryo growth is that of experimentation with artificial media, that is, the cultivation of plant embryos *in vitro*. It is generally true that any plant embryo, if old enough at the time of excision, may be successfully cultivated on a medium containing only water, agar, minerals, and sugar. Conversely, it has been found that the embryos of all species which have been tried, if young enough when excised, will fail to grow on any artificial medium now known. The experiments to be discussed here concern embryos between these two age limits. Van Overbeek, Blakeslee, and Conklin (11,12) found that growth of immature *Datura* embryos was benefited by the addition to the medium of an arbitrary mixture of growth factors including glycine, a number of vitamins, and several other organic nutrients. These supplementary substances, however, did not aid in the growth of embryos which were less than about .5 mm. long when excised. These still failed to develop. The further addition to the medium of unautoclaved coconut milk, a convenient source of endosperm, made it possible to grow undifferentiated embryos as small as .1 mm. long. Further evidence led to the conclusion that there are two factors present in coconut milk: a heat stable factor (probably auxin) causing growth but not differentiation, and a heat labile factor which induces differentiation. The material responsible for the latter effect has not been identified, but Blakeslee and Satina (1) have since found that a factor of comparable activity is present in malt extract. Haagen-Smit and co-workers (6), testing these findings on other material, found that coconut milk was not a limiting factor for the growth of excised maize embryos. Embryos

more than .3 mm. in length did not require coconut milk for continued growth *in vitro*, while smaller embryos failed to develop even if the medium were supplemented with coconut milk. This study indicates that the requirements for embryo growth vary among different plant species.

Curtis (4) found that the growth of embryos of many orchid species was better and more regular when peptone or nucleic acid were present in the medium. Nucleic acid from yeast was also shown by Kent and Brink (7) to be favorable for the growth of barley embryos which were too young to develop in its absence. The components of the substance which are active in this case have not been determined, but they are known to be heat stable.

Sanders and Burkholder (9), working with *Datura* embryos, concentrated their attention on the effects of various amino acids, and they showed that casein hydrolysate or a mixture of 20 amino acids markedly improved the growth and differentiation of the two species studied. The addition of individual amino acids and mixtures of a few generally resulted in much poorer growth than that occurring on the more complete mixture. Some of the individual amino acids or small groups of them caused modifications in the proportions of the embryos, increasing or decreasing the cotyledon size and increasing the number of root primordia, and sometimes premature root growth resulted. The amino acids in the medium thus appeared to influence both the growth and differentiation of *Datura* embryos. Spoerl (10), in his studies on orchid embryos, tested the effects of 19 amino acids as nitrogen sources, and found that most of them inhibited growth. Arginine, however, supported good growth of embryos from unripe seeds, while aspartic acid proved to be a satisfactory nitrogen source for older embryos.

In a study of the effect of casein hydrolysate on the growth of immature *Hordeum* embryos, Ziebur and co-workers (13) found that they could not duplicate the effect of this product with a mixture of merely amino acids. Casein hydrolysate has a clear-cut, two-fold effect on barley embryos: it prevents germination, and it supports embryonic growth. It was found that both parts of this effect could also be produced by supplementing the medium with amino acids, inorganic phosphate, and sodium chloride, all of which are components of commercial casein hydrolysate. Table 1 compares the effects of casein hydrolysate and of its three components, used singly and all together. The embryos

TABLE 1

The effects of various components of casein hydrolysate, tested singly and all together

Treatment	Osmotic pressure of medium (Atm.)	Number embryos	Average score	Average wet weight	Average dry weight	Average percent dry matter
Control	1.3	11	276 ± 52*	154 ± 22*	103 ± 15*	9.3 ± 1*
1) Amino acids	3.3	17	57 ± 8	102 ± 9	106 ± 6	16.3 ± 1
2) KH₂PO₄ 0.013 molar	2.0	16	156 ± 24	163 ± 20	14 ± 12	15.2 ± 1
3) NaCl 0.13 molar	7.1	17	84 ± 3	84 ± 11	85 ± 8	15.2 ± 1
4) (1-2) (1-3)	9.7	17	6 ± 3	40 ± 4	72 ± 6	25.9 ± 1
Casein hydrolysate 1%	9.7	13	4 ± 2	47 ± 5	88 ± 10	26.3 ± 1

*Standard error.

on the control medium did not continue their embryonic growth, but germinated in about three or four days, forming seedlings which are abnormally small and spindling. This type of growth is indicated when the shoot height is large and the percentage of dry matter is small (around 10 per cent). If 1 per cent of casein hydrolysate is added to the medium, very young embryos fail to germinate and older ones are retarded so that the shoot height is reduced relative to the control; the wet and dry weights are also reduced, but the percentage of dry matter is high. The effect of the amino acid mixture containing phosphate and sodium chloride is indistinguishable from that of the casein hydrolysate. Each component alone, however, fails to duplicate the effect of the complete mixture. The added phosphate reduces the height slightly, while the amino acids and sodium chloride are more effective in this respect. Note that, in general, the shoot height is reduced on media with high osmotic pressure. The percentages of dry matter of embryos grown on media containing each of the three supplements singly are intermediate between those of the control and the complete mixture.

A similar experiment, using the components in combinations of two, showed that none of the three possible pairs reproduced the results given by the casein hydrolysate, although the combination of amino acids and sodium chloride with an osmotic pressure of about nine atmospheres caused almost complete suppression of germination. Experiments with high concentrations of sucrose or mannitol demonstrated that the inhibition of germination could be attributed to the high osmotic pressure of the medium, as shown in Table 2. On all three media of high osmotic pressure the shoot height is low and the percentage of dry matter is high. It may be concluded then that the germination-inhibiting effect of casein hydrolysate is due to its osmotic pressure, for which the amino acids and sodium chloride are mainly responsible. The rapid embryonic growth which takes place on the casein hydrolysate medium is greater than that occurring on the media supplemented with sucrose or mannitol; the wet weight of the embryos on the casein hydrolysate medium is the greatest, and the dry weights on the casein hydrolysate and sucrose media, respectively, are greater than on the mannitol medium. Mannitol is nutritionally inactive for barley embryos. It is thought, therefore, that these weight differences result from the fact that casein hydrolysate and, to a lesser extent, the additional sucrose serve as nutrients as well

TABLE 2

The effects of high concentrations of casein hydrolysate, sucrose, and mannitol

TREATMENT	OSMOTIC PRESSURE OF MEDIUM (ATM.)	NUMBER EMBRYOS	AVERAGE SHOOT HEIGHT	AVERAGE WET WEIGHT	AVERAGE DRY WEIGHT	AVERAGE PER CENT DRY MATTER
Control (2% sucrose)	1.3	11	398 ± 5	254 ± 12	142 ± 9	11.4 ± .7
Casein hydrolysate 1%	9.7	15	0	73 ± 10	105 ± 6	32.3 ± 2.3
Sucrose 12.8% (additional)	9.7	14	1 ± 1	44 ± 3	101 ± 6	46.7 ± .9
Mannitol 6.8%	9.7	15	2 ± 1	32 ± 2	55 ± 4	35.8 ± 1.8

as osmotic agents. The experiments with pairs of components lead to the further conclusion that the nutritional effect of casein hydrolysate may be attributed to the amino acids and phosphate. The results as a whole suggest that one is dealing here with another case where the determination of the amount and the type of development is not mainly dependent on one specific growth requirement of the embryo, but may result from an interplay of both nutritional and physical factors.

In summarizing this discussion it will be realized that the field of plant embryo growth is relatively undeveloped. A number of workers have had a variety of results with different materials, and it is difficult to draw from their data any conclusions as to the important factors which in general influence the growth of plant embryos. Histological studies have contributed an understanding of the probable functions of the various seed parts in supporting and controlling the growth of young embryos, but the exact chemical nature of the substances involved is not known. Experiments on the inhibition of seed germination have led to the discovery of a variety of ways in which the development of older embryos may be controlled in nature. *In vitro* studies of the embryos of a number of different species have shown that at early stages embryos require for their nutrition not only minerals and sugar, but also in some cases vitamins, amino acids, nucleic acid, and unknown materials occurring in coconut milk or malt extract. And the pattern of growth followed by an embryo may be influenced not only by the known growth hormones, but also by amino acids and such other factors as osmotic pressure. Future research in this field should include more of the exploratory work just reported, but especially should attempts be made to relate the effects of an apparently diverse group of factors all of which play an influential role in the growth of plant embryos.

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Growth Substances and the Formation
of Buds in Plant Tissues

FOLKE SKOOG AND CHENG TSUI

THE analysis of developmental processes of plants, particularly in relation to the role of growth substances, is beset by difficulties of many kinds, but perhaps mainly from two sources. One is the continuous change in composition and rates of synthesis of growth factors in the tissues, and another is the complex influences of each part on the growth of other parts and on the development of the plant as a whole. In fact, from comparisons based on mere inspection of normal and pathological plant materials, one may conclude that normal development, as contrasted with simple growth, must depend more directly on correlative processes, often of an inhibitory nature, than on the actual synthesis of new cell materials. For experimental purposes, therefore, it seems necessary to start with simple materials with limited capacities for autotrophic growth and differentiation.

This report deals primarily with the chemical induction of organs, especially of buds, in parenchyma tissue and excised segments of stems and roots grown *in vitro* on media of known chemical composition. Some conclusions drawn from these experiments which may have a general bearing on growth and morphogenesis in plants will also be discussed. The intimate connection between this topic and the general problem of the correlative action of growth substances in plants will be apparent.

Experiments with tobacco callus cultures.—During the period 1937–1940 several investigators (3,8,13,12,10) obtained evidence of different

kinds which provided indirect support for the view that auxin may act as a coenzyme. If this is true, it means that auxin must act in one or perhaps several, but nevertheless definite, metabolic systems. Further-more, from the fact that quantitative growth responses to applied indoleacetic acid are obtained only over a restricted range of concentra-tions, it may be deduced that other components of this "auxin reaction" system must also be present in relatively limited quantities in the plant. It was of interest, therefore, to determine what this system and its other components might be. A possible experimental approach to this problem became available with the development by White (14) of callus tissue cultures which could be grown indefinitely on a medium of known composition. White found that callus of the hybrid *Nicotiana glauca* × *N. langsdorffii* grown in submerged cultures would differentiate to pro-duce numerous buds (15), and one of us found (9) that the addition of indoleacetic acid (IAA) or naphthaleneacetic acid (NAA) to the culture medium would completely prevent the initiation of buds. Even low concentrations (0.1 mg./l. or less), which did not retard the rate of increase in fresh or dry weights of the tissues, effectively prevented bud formation, whereas higher concentrations inhibited also the rate of growth of the callus. In short, the tobacco tissue cultures have a very sensitive response to IAA and are a favorable material for the testing of various organic compounds which might normally interact with auxin. On the above assumption of an auxin reaction system, such compounds should counteract the inhibiting effect of IAA on bud formation and at the same time should increase the growth of the callus in media with added IAA.

Substances were selected for testing which are, or might be, required in relatively large amounts in respiration. Tests were first carried out with the four-carbon dicarboxylic acids, malic and succinic acids. The results were negative. Such effects on bud formation as were obtained by the addition of these acids could be ascribed entirely to lowering of the *p*H of the medium. A careful study of the relation of *p*H to bud forma-tion (16) showed that the optimum was in the range from 4 to 4.5 (9).

The effects of increased concentrations of phosphate and sucrose were tested with positive results. The data from one experiment are sum-marized in Table 1. It may be seen that about half the callus pieces form buds in the control nutrient solution and that none form buds in the cultures with added IAA. With increased KH_2PO_4 and sucrose contents,

on the other hand, bud formation occurs also in the presence of IAA and up to about the same extent as in the control cultures without added IAA. In these experiments the concentration of $Fe_2(SO_4)_3$ was also increased to maintain available iron in the medium. Control series with increased $Fe_2(SO_4)_3$ alone were negative, so that the effect on bud formation is ascribed to the KH_2PO_4. *

TABLE 1

Effect of indoleacetic acid, and changes in concentrations of KH_2PO_4, $Fe_2(SO_4)_3$, and sucrose on growth and bud formation in tobacco callus *in vitro*. Initial pH adjusted to 5.0. Cultures started 12/22/41. Final measurements 3/19/42

	CONTROL		4FE4P		4FE8P		4FE8P2S	
	INDOLEACETIC ACID MG./L.							
	0	1.0	0	1.0	0	1.0	0	1.0
No. of cultures	17	17	17	18	18	17	17	16
Mean fresh weight in mg.	54	42	65	42	47	49	50	49
Mean dry weight in mg.	3.4	2.6	4.1	2.6	2.9	3.1	3.1	3.1
No. of cultures forming buds	9	0	8	3	8	9	10	9
Per cent forming buds	53	0	47	17	45	53	53	56

4 Fe = $Fe_2(SO_4)_3$ 10 mg./l.; 4 P and 8 P = KH_2PO_4 50 and 100 mg./l., and 2 S = sucrose 40 g./l.

The effectiveness of phosphate in counteracting the inhibitory effect of added auxin suggested that organic phosphates might be limiting components of the auxin reaction system. Since an important influence of phosphate in respiration is through its role in purine metabolism, it seemed desirable to test phosphorylated adenine derivatives. None were immediately available and, therefore, adenosine in combination with inorganic phosphate was first tried. The results of one experiment are shown in Figure 1. It may be seen that either adenosine or NAA added singly reduces the growth of the callus, but the two added in combination give as good or better growth than the controls. However, no buds were obtained on either control or treated tissues in this experiment. In

*Recently R. W. Howell has compared the effects of NaH_2PO_4 and KH_2PO_4 on bud formation in tobacco stem segments. He finds both equally active so that the effect of the salts may be ascribed definitely to the $(H_2PO_4{}^-)$ radical rather than to the accompanying cation.
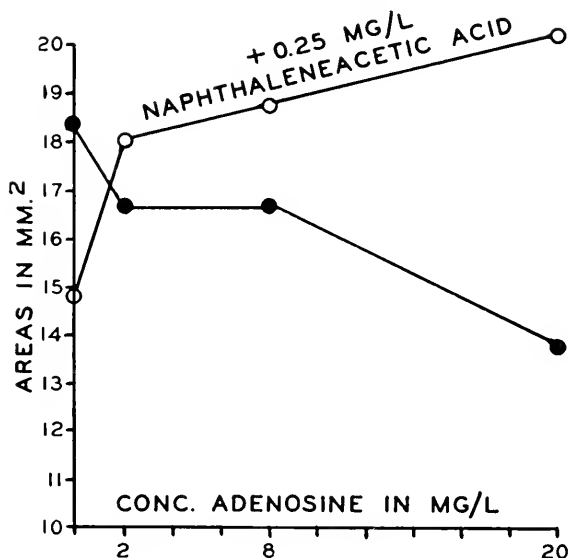
Figure 1. Effect of concentration of adenosine on the growth of tobacco callus *in vitro* with and without addition of 0.25 mg./l. NAA. Exp. #14 started 12/24/43. Harvested 3/16/44. Data of 32 cultures for each treatment.

later experiments increases were obtained in the number of callus tissues forming buds as illustrated in Table 2.

The addition of adenine similarly was found to stimulate the formation of buds, and adenine is, in fact, more active than corresponding concentrations of adenosine (Table 3, compare also Fig. 7).

The above results show that the inhibiting effect of auxin on bud formation is reversibly counteracted and that the growth rate of callus in the presence of added IAA is increased by the addition of adenine or adenosine and phosphate. The results suggest, therefore, that auxin may exert its effect on growth through an action in one or more phosphorylation systems.

Experiments with tobacco stem segment cultures.—The effects obtained with treatments of IAA, adenine, and its derivatives on callus cultures have been confirmed and the results considerably extended by experiments with excised stem segments of tobacco.

Young stems of variety Wis. #38 were surface sterilized, sectioned, and cultured on White's nutrient medium as described by Skoog and Tsui (11). This material was found to give clear-cut and reproducible

TABLE 2

Effect of adenosine on bud formation in tobacco callus cultures. (White's medium, 3/4 strength. Experiment started 9/23/43, measurements made 1/1/44.)

MEASUREMENTS	ADENOSINE CONC. IN MG./L.					
	0	0.5	2.0	10.0	50.0	2.0*
Number of cultures	52	33	37	40	36	38
Number forming buds	2	2	2	6	7	6
Per cent forming buds	4	6	5	15	20	16

*Cultures in the last column received increased concentrations of the following components of the basic medium: KH_2PO_4, 5 times (62.5 mg./l.); $Fe_2(SO_4)_3$, 3 times (7.5 mg./l.); and sucrose 2 times (40 g./l.).

responses to treatments with adenine and IAA or NAA. It was, therefore, chosen for detailed studies of the effects of the above compounds on growth and organ formation. These include measurements of effects of added growth substances and nutrients as well as quantitative analyses of associated changes in composition of various constituents and nutrients within the tissues. The work is still far from complete, but the results reported here are based on our present experience with about ten thousand cultures.

Representative results of the effects of the different treatments with relatively high adenine and low IAA concentrations on growth are shown in Figure 2, and results of anatomical studies, carried out by Dr. Sterling, on these tissues at successive stages of their growth are summarized by the diagrams in Figure 3.

TABLE 3

The effect of adenine sulfate on bud formation of tobacco calli obtained in first transfer (on 6/18) from stem internode segments cultured for 29 days. Cultures started in liquid media 6/18/48. Measurements 7/21/48

CONCENTRATIONS OF ADENINE SULFATE	NUMBER OF CALLI OBSERVED	NUMBER OF CALLI FORMING BUDS	NUMBER OF BUDS FORMED
0 (control)	27	0	0
2.5 mg./l.	30	4	4
5 mg./l.	30	6	10
10 mg./l.	21	7	14

No root formation occurred.

CONTROLLED GROWTH IN TOBACCO STEM SEGMENTS

XYLEM
PHLOEM
PARENCHYMA
CALLUS
CAMBIUM
PRIMORDIAL MERISTEM

C   CONTROL
IAA INDOLEACETIC ACID
Ad   ADENINE SULFATE
LS   LONGITUDINAL SECTION
XS   CROSS SECTION

NORMAL XS

NORMAL LS

4 WEEKS

4 WEEKS

2 WEEKS

C
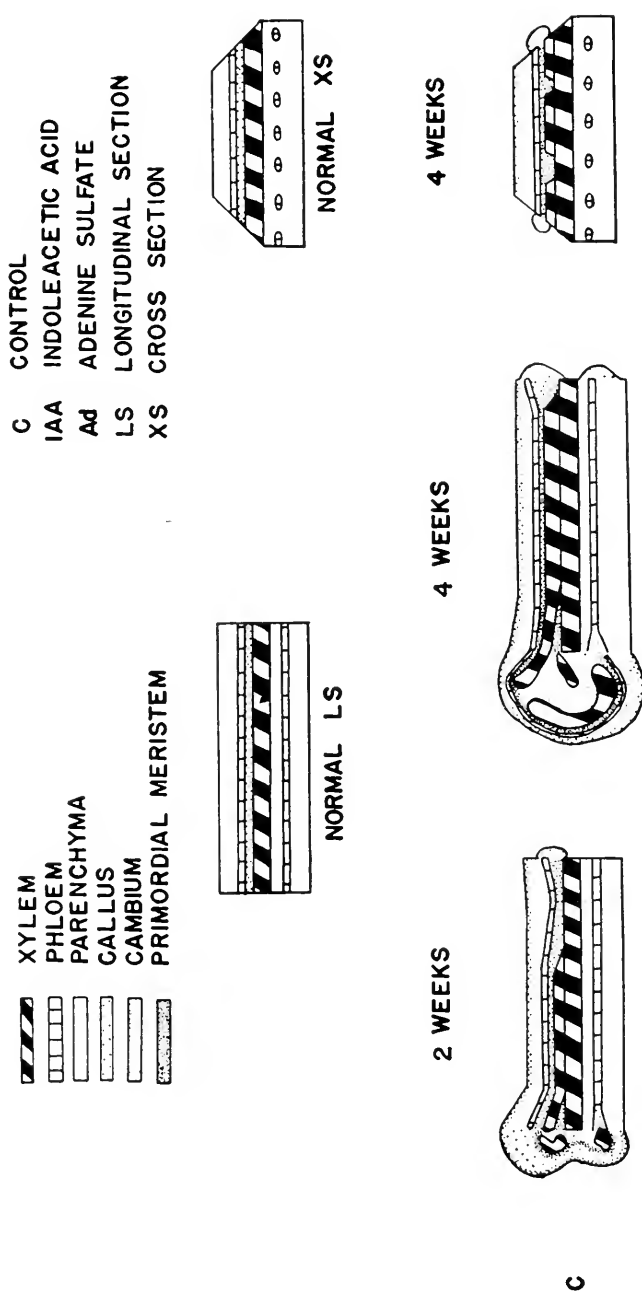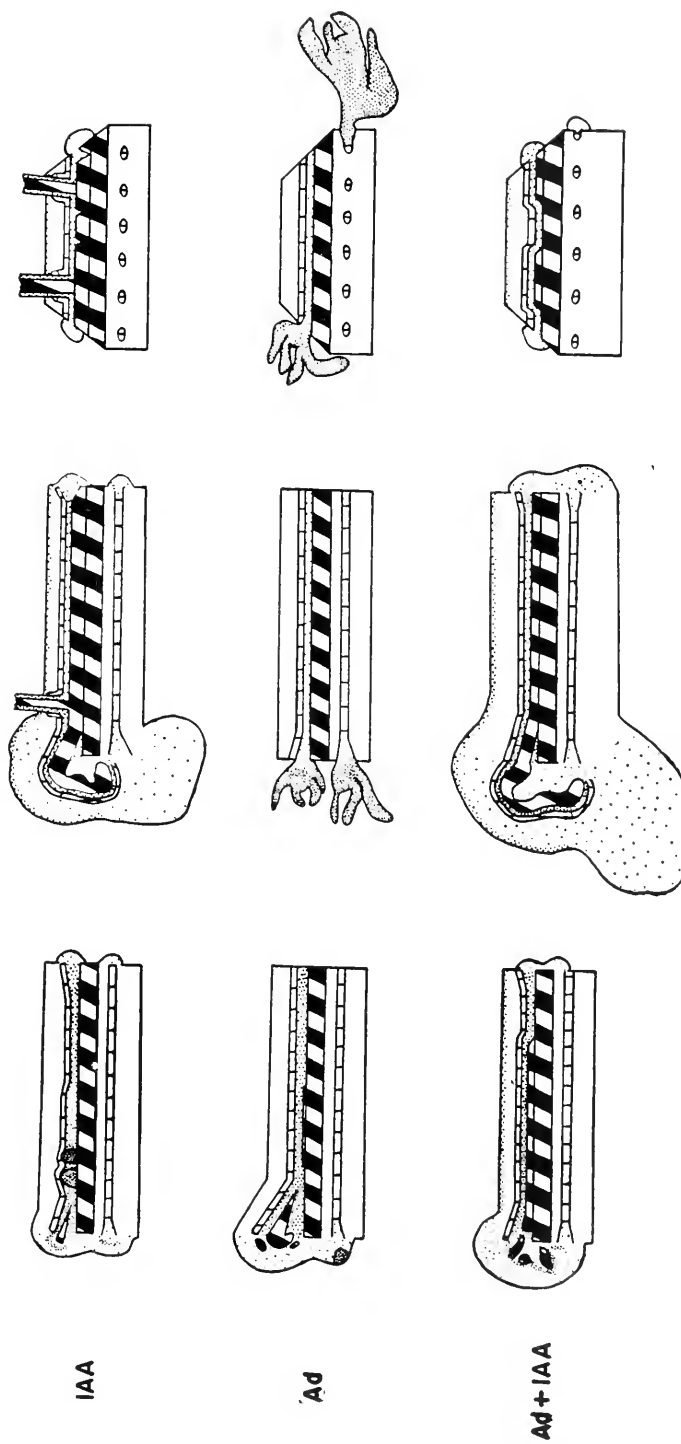
Figure 3. Diagrams showing anatomical features of growth and organ formation in tobacco stem segments treated as in Figure 2. Data obtained by C. Sterling from tissues of Exp. #A (started 7/29/48) and Exp. #E (started 6/19/49).

Growth of control segments.—The ability of the stem segments to grow on the basal medium is a function of their size and of the vigor of the plants from which they were taken. Pieces 3 x 2 x 2 mm. or less in size ordinarily remain alive for several weeks but do not grow appreciably unless they are supplied with IAA. Larger pieces (7 x 5 x 2 mm.) which were selected as standard material for these and all subsequent experiments form masses of callus at their basipetal ends within two to four weeks in culture. After longer periods (6 to 10 weeks) a few pieces (5 per cent on the average) may form a bud or root or both, but growth is restricted mainly to the formation of callus, which starts from the cambial region of the basipetal surface. This callus may be subcultured on the same medium with added IAA, but, at least in most cases, neither the original stem tissue nor the first transfer of callus will continue to grow on the basic medium without IAA.

Effects of adenine treatments.—When adenine is added to the medium the formation of callus is less than in controls, but a very striking formation of buds occurs. These buds grow out most abundantly along the lateral edges and especially from the apical halves of the segments with relatively lower auxin content, but also to some extent from the basipetal halves and attached, newly formed, callus. As indicated in Figure 3, Dr. Sterling has found that they originate from parenchyma formed by the cambium and from both external and internal phloem regions. The number of pieces forming buds in a given treatment and the number of buds per piece both increase as functions of the concentration of adenine in the medium up to about 50 mg./l. Higher concentrations are often toxic. With optimal concentrations, generally between 90 and 100 per cent of the segments form buds. The effects of concentration of adenine and time of culturing are illustrated in Figure 4. In this experiment the highest average number of buds per segment was 2.7 after twenty-eight days. In an experiment with tissues from field-grown plants, however, an average of about 20 buds per segment and extremes of 35 to 40 buds per segment have been obtained. The number of buds obtained is not necessarily the maximum number that can be produced by the tissue, because as one or a few buds start to develop, they produce auxin in sufficient quantities to raise the concentration in the original stem tissues, and thus tend to retard or prevent further bud formation. The buds which form early may develop several leaves, but, unless root formation occurs, the buds are incapable of continued growth. If they form roots

and are transplanted, however, they grow into normal mature plants.

Effects of IAA or NAA treatments.—Even though there are important quantitative differences in growth responses to IAA and NAA, the general effects of these substances in the present experiments are closely enough alike, so that they can be considered together. The addition of either IAA or NAA to the medium markedly stimulates the growth of
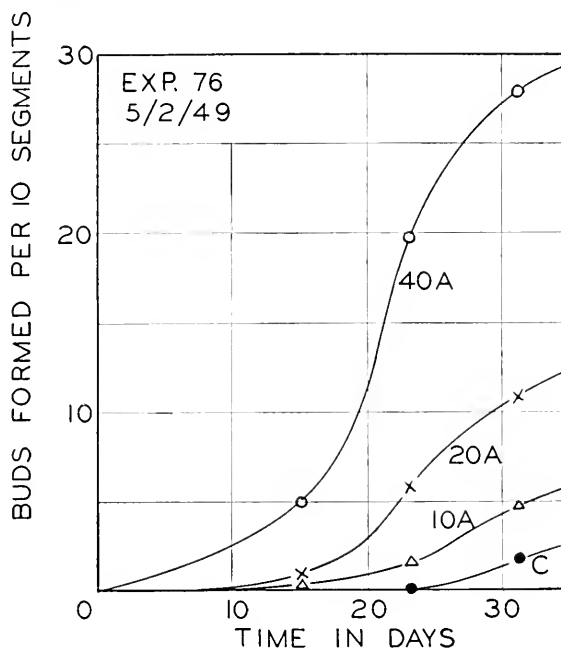
Figure 4. Effect of concentration of adenine sulfate on bud formation in tobacco stem segments. Exp. #76 started 5/2/49. Curves in order from the top represent treatments with 40, 20, 10 and 0 mg./l. adenine respectively. Initial pH 4.0, KH_2PO_4 concentration 37.5 mg./l.

the tissues through both enlargement of existing cells and the formation of callus over wider areas of the segments.

Significant increase in callus growth and decrease in bud formation may be obtained with as little as 0.02 mg./l. and maximum effects with concentrations in the range from 1 to 10 mg./l. These substances also induce the formation of roots as indicated in Figures 2 and 3, which effect is comparable to that known to occur in cuttings.

Effects of adenine plus IAA or NAA.—Combined treatments with

adenine and IAA or NAA lead to marked enlargement and cell division in the tissues of the stem segments particularly of the pith and superficial cortical layers. However, the relative growth of the different tissues depends on the relative concentrations of the two substances.

The extent of organ formation is also dependent on both the concentrations and proportions of adenine and auxin. For example in the treatment shown in Figure 2, with high adenine and relatively low IAA, there is very rapid growth of the original tissues and of new parenchyma, but there is no organ formation. With higher concentrations of IAA, root formation occurs even though it is much less than would be obtained from the same concentrations of IAA in the absence of adenine. The results obtained with high adenine and only minute concentrations of IAA are illustrated in Figure 5. Here the formation of buds is restricted to the acropetal ends of the segments and roots usually form later at the bases of the buds and or at the basipetal ends of the stem segments.

In general, therefore the results indicate that under the conditions of these experiments the types of growth and organ formation which occur are determined by the concentrations of auxin and adenine supplied in the nutrient medium. Relatively high auxin concentrations favor the formation of roots and prevent the formation of buds. High adenine concentrations favor the formation of buds and decrease the extent of root formation. With both substances added together in proper concentrations it is possible to get marked stimulation of growth without organ formation.

Comparative experiments with horse radish and carrot root segments.—The results obtained with tobacco stem segments indicate that the morphogenetic response to treatments with adenine and IAA must vary with the tissue composition. Hence considerable variability in responses is to be expected between different organs of the same plant and particularly between different species. Tests were, therefore, carried out with horse radish and carrot root tissues which can be conveniently cultured. The former material was selected because it has a marked capacity to form adventitious buds and for this reason has been used extensively in work on regeneration. As shown in Figure 6, control segments of horse radish root grown on the basal medium produced on the average one bud per segment. In the presence of adenine the number of buds was doubled and their appearance was hastened. The presence of IAA even in low concentrations sometimes completely prevented the formation of

Figure 2. ...

Figure 5. Effects of treatments with high adenine combined with very low IAA concentrations on bud formation in tobacco stem segments. Exp. #42 started 9/17 48. Photographed after 115 days. Treatments from left to right: Control; adenine sulfate 40 mg. l.; do. plus 1γ l. IAA; do. plus 5γ/l. IAA. Initial pH 4.0; KH₂PO₄ 12.5 mg./l.

buds and in all cases markedly retarded the appearance of buds. How-
ever, when adenine was supplied together with IAA in the same con-
centrations as of each substance above, bud formation was restored, in
this experiment, to the same value as in the treatments with adenine
alone. These results are in general agreement with the data for tobacco,
even though the inherent capacity to form buds was greater and the
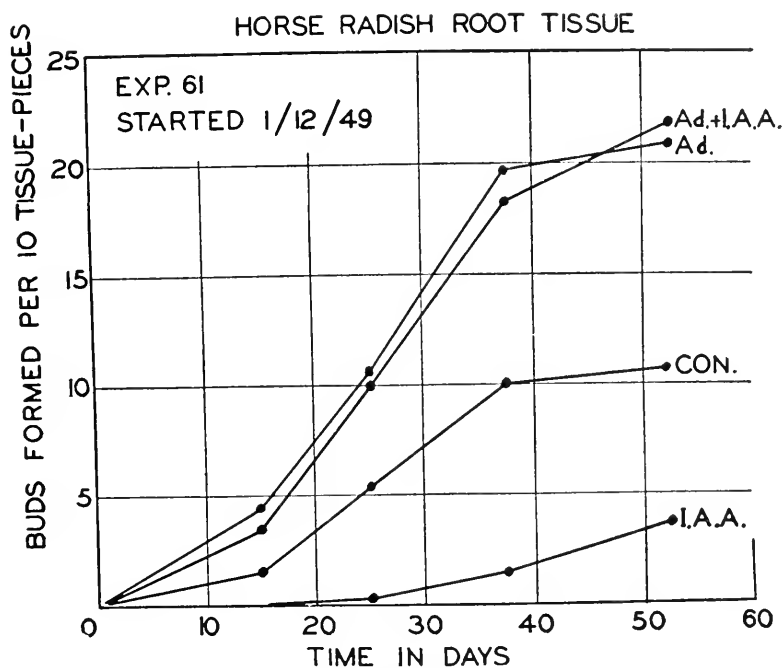
Figure 6. Effects of treatments with adenine sulfate and IAA on bud
formation in horse radish root segments. Exp. #61 started 1/12/49. Initial
pH 4.0; KH_2PO_4 37.5 mg./l.

capacity to form roots directly from the original tissue was less in the
horse radish. These differences are strikingly correlated with the relative
auxin contents of the tissues of the two species (Tables 5 and 6).

Carrot tissues responded differently. The growth of the original seg-
ments, depending on their size and morphological make-up probably
as well as on the strain, was either stimulated or inhibited by low con-
centrations of IAA in different experiments, and was stimulated by higher
concentrations. The highest concentrations (10 to 100 mg./l.) induced

root formation. Adenine had relatively little effect on growth when supplied alone, but it completely counteracted the inhibiting effect, when it was obtained, from low concentrations of IAA. No bud formation resulted from treatments of the segments. However, in subcultures of the callus produced on the segments, roots were formed in response to IAA treatments, and a few transfers formed buds, but not exclusively in cultures with adenine treatment. In tissues from all three species, therefore, a very definite interaction between adenine and IAA was demonstrated, but the quantitative requirements for a particular type of growth or organ formation were very different.

Specificity of adenine for bud formation.—In the case of auxins we know that a large group of related compounds with certain structural features in common have similar effects on cell elongation and also on root formation. It was of interest, therefore, to determine the specificity of adenine in its effect on bud formation. As shown by the curves in Figure 7 adenine derivatives such as adenylic acid and adenosine are active. Also guanine, though it acts more slowly, may be nearly as effective as adenine. However, xanthine and the pyrimidine, uracil, are either completely inactive or have a very low activity. Preliminary tests with cytosine indicate that this compound may possess some activity. On the other hand, the buds which were produced in tissues treated with cytosine appeared so much later than from treatments with adenine, that the effect of this substance may be less direct. The results are as yet fragmentary but suggest that the entire purine nucleus together with either substituted NH_2 groups in the 2 or 6 position, or else the absence of keto groups in both these positions is required for high activity.

Arginine, which has been reported to substitute for adenine in the phosphate energy transfer in invertebrate tissues, has also been shown to promote the growth of *Avena* coleoptiles and pea stem segments (1,4). It also promotes growth of tobacco tissues under the conditions of our experiments, but, in our limited experience with the tobacco tissues, arginine has a general growth-promoting effect which is quite distinct from the effect of the active purines. The specificity of adenine and structurally related compounds for bud formation may well be compared with the specificity of IAA, and other compounds with auxin activity, for root formation. Still, neither group of compounds can be designated as specific organ-forming substances, since both are unquestionably essential for growth of all cells and tissues. Furthermore,
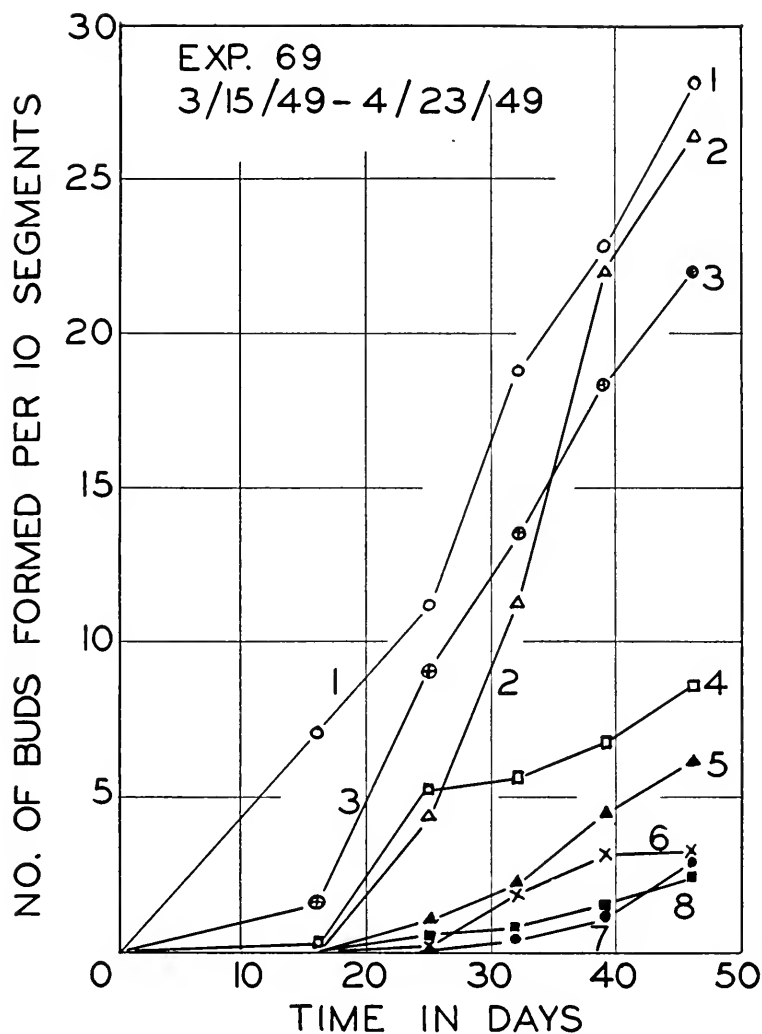
Figure 7. Relative activities of different purine derivatives and uracil on bud formation in tobacco stem segments. Exp. #69 started 3/15/49. Initial pH 4.0, KH$_2$PO$_4$ 12.5 mg./l. Curves: 1. adenine sulfate; 2. guanine; 3. adenylic acid; 4. nucleic acid; 5. adenosine; 6. xanthine; 7. uracil; and 8. control. All substances added in 0.00025 molar conc. except nucleic acid which was 85 mg./l.

even though they may be limiting factors for the formation of buds and roots respectively under normal conditions as well as under a given set of experimental conditions, it is obvious that different conditions could be selected, where organ formation would be limited by other factors.

Effects of inorganic nutrients.—While White's nutrient medium was originally devised for continued culture of excised roots, it contains all necessary ingredients required for continuous growth of tobacco callus cultures, but certain quantitative modifications in mineral components result in more rapid growth rates of the tissues. It is a question, therefore,

TABLE 4

Effect of adenine sulfate added to the nutrient medium at different pH values on the formation of buds in stem segments of tobacco. Exp. 39. Started 8/5/48

ADENINE SULFATE CONCENTRATIONS IN MG./L.	INITIAL pH OF MEDIUM	NO. OF TISSUES CULTURED	BUDS FORMED PER 10 SEGMENTS AFTER		
			18 DAYS	28 DAYS	60 DAYS
0	4.0	24	0	0	0
40	4.0	27	4.8	10.7	15.9
40	5.0	21	0	1.9	4.7
40	6.0	24	0	2.0	5.0
40	7.0	18	0	2.2	3.8

to what extent changes in mineral composition might also affect the capacity of the tissues to form organs. So far only a few of the many possible variations in nutrient composition have been tested in tobacco stem segments. Thus, a marked effect on pH on bud formation in response to added adenine has been established. As indicated in Table 4, adenine is effective in inducing bud formation in the range from pH 4 to pH 7 which was tested, but acid pH is highly favorable. Since acid pH also favors bud formation of callus tissue in submerged cultures without added adenine, it may be assumed that the pH effect is not entirely, if at all, on the adenine uptake by the tissues.

Similarly, increases in phosphate supplies in the medium increase the effectiveness of the adenine treatments. As shown in Figure 8, a threefold increase in phosphate content (from 12.5 mg./l. to 37.5 mg./l.) causes about twice the number of buds to be formed per segment treated with 40 mg./l. adenine. However, a ninefold increase in phosphate is evidently

above the optimum requirement and no better than the standard amount.

Variations in potassium concentrations as stated above appear to have no effect on bud formation. Different NO_3^- levels have been tested only in callus cultures not supplied with adenine. No significant effect on the capacity of the tissues to form buds was obtained in these experiments. Other components may have some effect but have not been studied in detail.

Interactions of IAA, adenine, ribose, and phosphate.—Since the metabolically active nucleotides contain ribose as well as phosphate combined with adenine it was of interest to test effects of ribose on growth and
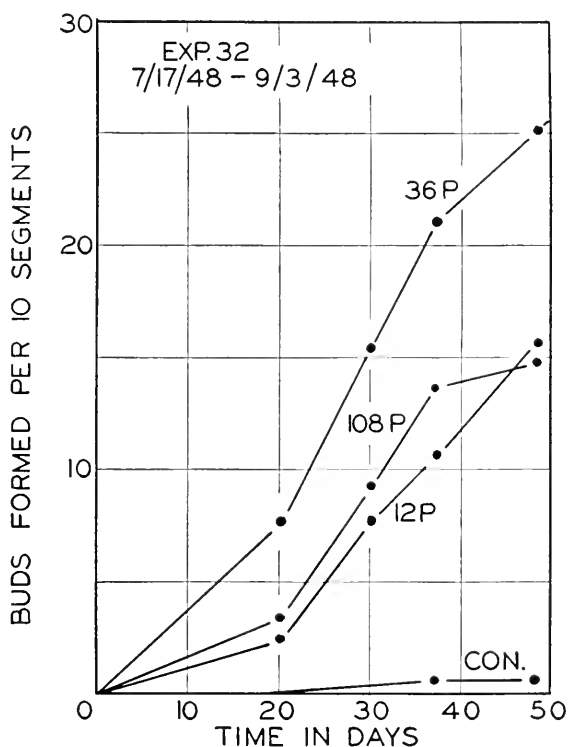
Figure 8. Effect of phosphate concentration on bud formation in tobacco stem segments treated with adenine sulfate. Exp. # 32. Started 7/17/48. Initial *p*H 4.0. Curves: 1. control without adenine, KH_2PO_4 12 mg./l.; 2. adenine sulfate 40 mg./l.; KH_2PO_4 12.5 mg./l.; 3. same as 2 except KH_2PO_4 37.5 mg./l.; 4. same as 2 except KH_2PO_4 112.5 mg./l.

organ formation. So far our results on ribose are restricted to tests on growth of callus supplied with adenine. IAA. and different levels of phosphate singly and in different combinations. Interestingly enough, in the lower concentration range ribose may have a growth-promoting effect. but even in concentrations as low as 10 mg. l. it may be strongly inhibitory. as is IAA. The two substances together exert an even stronger inhibitory effect on growth. However, in the presence of adenine this inhibition is counteracted in all cases. and. in fact. with low ribose concentrations more rapid growth. as measured by average increases in fresh and dry weights of the tissues. have been obtained than from the addition of adenine or adenine and IAA in the absence of ribose. However. the additional increase in growth obtained from ribose in the presence of adenine and IAA. has so far been too small to be definitely significant. In spite of this. the results obtained with combinations of any two or all three components of the nucleotides strongly suggest a close relationship between them and auxin in their effect on growth.

Analyses of tissue composition.—Analyses of tissue composition including "free" and bound auxin. adenine. phosphate fractions. and changes in these components with treatments leading to different types of growth and organ formation are in progress. In general it may be said that the readily extractable auxin content in the tobacco stem segments decreases rapidly from the original level. and a gradient with relatively higher concentrations present in the basal half than in the apical half is established. A return to higher auxin levels occurs after buds have formed and. therefore. becomes most marked at first in segments treated with adenine. However. rapid growth of the segments themselves is also associated with increased auxin content. Representative data are shown in Table 5. The effects are particularly marked in horse radish segments (Table 6 whose auxin content is lower than in tobacco at the start and higher after buds have been formed. These changes in auxin content. therefore. run parallel with the growth and type of organ formation which occur. Low auxin levels are associated with bud formation and high auxin levels with root formation. It is probable. therefore. that the number of buds forming on a segment is limited by the auxin production in the buds which first grow out. Due to difficulties in obtaining quantitative estimates of auxin concentrations in carrot tissues no reliable data have yet been obtained on this species.

From the results presented above. it might be assumed that adenine

TABLE 5

Auxin content in tobacco stem segments cultured on media with adenine and
IAA. Expt. C. Cultures started 2 18 48. Tissues extracted 2 hours with ether

TREATMENTS	ETHER-EXTRACTABLE AUXIN IN γ IAA EQUIVALENTS KG. FRESH TISSUE					
	2 DAYS		14 DAYS		28 DAYS	
	TOP	BASE	TOP	BASE	TOP	BASE
Control	14.3	15.8	3.3	4.0	5.7	10.7
Adenine sulfate 40 mg. l.	14.3	15.8	2.0	4.0	4.0	16.0
Indoleacetic acid 0.04 mg. l.	14.3	15.8	3.1	2.6	5.5	7.1
Indoleacetic acid 0.04 mg. l. and adenine sulfate 40 mg. l.	14.3	15.8	2.8	2.9	9.3	10.1

is absorbed and combines with phosphate in the tissues and that the
content of organic phosphate would be affected by the external supplies
both of IAA and adenine. Experiments by Holm 6 have established
that treatments with IAA tend to increase the total phosphate content
of the apical halves of the tobacco stem segments over that in controls.
but tend to decrease the phosphate content of the lower halves. Addition
of adenine, on the other hand, has relatively little effect on the phosphate
content in the apical halves but tends to increase the concentration in
the basal halves. Adenine supplied in combination with IAA leads to

TABLE 6

Auxin content of horse radish root segments cultured on media with adenine
and IAA. Exp. 95. Started 2 24 40; initial pH 4.0. Phosphate 57.5 mg. l.
Tissues extracted 2 hours with ether

TREATMENTS	ETHER-EXTRACTABLE AUXIN IN γ IAA EQUIVALENTS KG. FRESH WEIGHT AFTER:		
	0 DAYS	14 DAYS	28 DAYS
Control	3.8	4.0	3.5
Adenine sulfate 40 mg. l.	3.8	3.2	12.1
IAA 0.02 mg. l.	3.8	2.0	7.3
Adenine sulfate 40 mg. l. and IAA 0.22 mg. l.	3.8	2.0	12.3

increases in the phosphate content of the segments and particularly counteracts the lowering in phosphate content which is apt to result from addition of IAA alone. However, the main increases in phosphate content obtained by the treatments have been in the inorganic fraction. Significant increases in organic phosphate fractions, therefore, either do not occur or the compounds formed are too labile to be detected in spite of all attempts with the several methods which were employed. In any case the results point to a sensitive equilibrium between auxin, adenine, and phosphate contents in the tissue, according to which phosphate uptake may be increased or decreased by addition of either one of the two organic compounds, depending on the relative supply of the other. Typical results of the phosphate content and distribution for different treatments and at successive stages of growth are shown in Figure 9, and the proportions of the total phosphate present in different fractions at the start and after 14 days of growth are shown in Table 7. Further evidence on the localization of the phosphate in the tissues is being obtained by isotope analysis. Data on adenine content and carbohydrate fractions are still too fragmentary to be interpreted.

Discussion and conclusions.—The evidence obtained on growth and organ formation in tissues cultured *in vitro* demonstrate that under the conditions of these experiments the relative growth of different tissues and the type of organ formation which will occur depend on the composition of the nutrient medium. Generally the application of IAA or NAA leads to rapid growth of parenchymatous tissues, to root formation, and to suppression of bud formation, whereas the application of adenine leads to bud formation. Clearly, however, these substances are not specific either for the formation or growth of particular organs. Both are required for all types of growth. The responses elicited by their application to the tissues depend on the proportions and concentrations in which they are supplied as well as on the proportions and concentrations of these and other essential nutrients present at the start or supplied during the growth period, through synthesis or from external sources.

In regard to the specific actions of auxin and adenine in growth the only new information provided by the present experiments is the definite interaction of these substances and other components of the nucleotides. This strongly supports the original assumption that auxin acts as a coenzyme. If we accept the known function of adenine phosphate
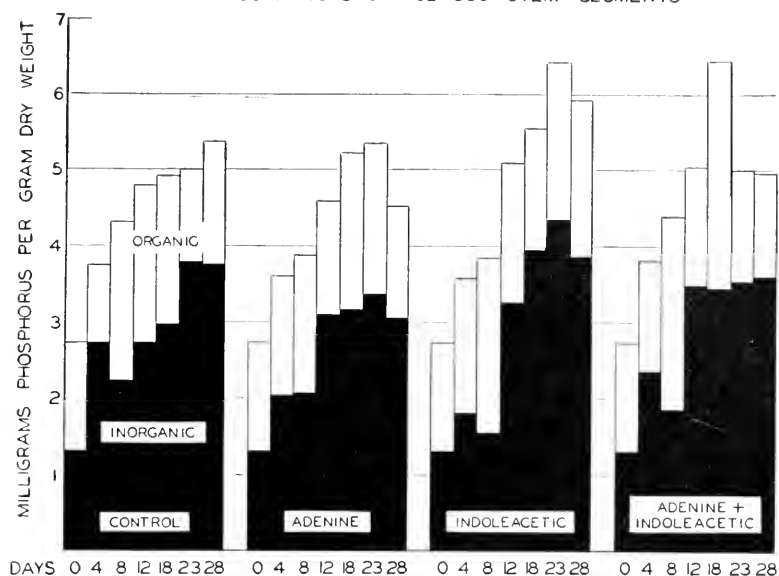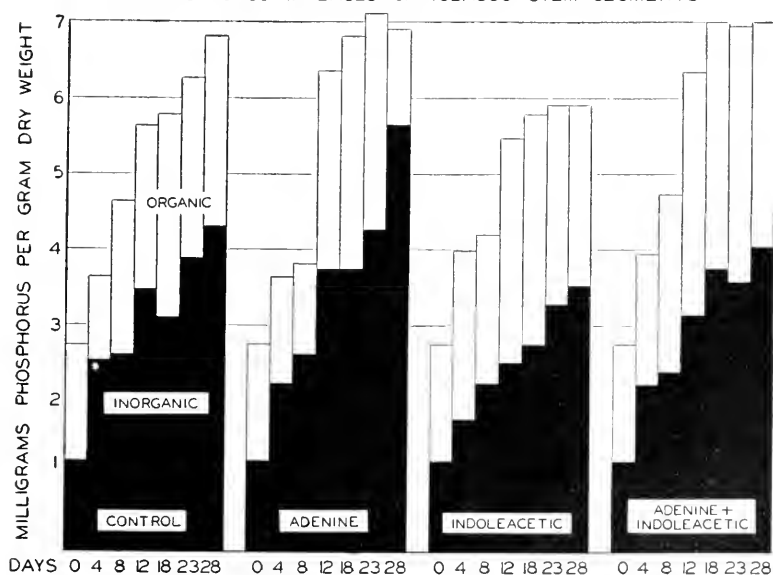
Figure 9. Effects of treatments with adenine sulfate and IAA on total, inorganic, and organic phosphate contents in tobacco stem segments. Exp. #G started 6/19/49. Initial pH 4.0.; KH_2PO_4 37.5 mg./l.; Adenine sulfate 40 mg./l.; IAA 0.04 mg./l. A. Upper halves of segments; B. Lower halves of segments. Shaded portion of columns are inorganic fractions. White portions are organic fractions. (Data from L. G. Holm.)

TABLE 7

A partial fractionation of the phosphorus compounds in tobacco internodal stem segments supplied with adenine and indoleacetic acid

| | 0 DAYS | | 14 DAYS | | | | | | | |
| | BEGINNING MATERIAL | | CONTROL | | ADENINE SULFATE (40 MG./L.) | | IAA* (0.04 MG./L.) | | ADENINE SULFATE (40 MG./L.) + IAA (0.04 MG./L.) | |
	μG./G. FR. WT.	% OF TOTAL	μG./G. FR. WT.	% OF TOTAL	μG./G. FR. WT.	% OF TOTAL	μG./G. FR. WT.	% OF TOTAL	μG./G. FR. WT.	% OF TOTAL
A. Total phosphorus	97.50	100.0	310.05	100.0	338.00	100.0	317.50	100.0	336.50	100.0
B. Inorganic phosphorus										
Acid-extractable fraction	34.20	35.10	194.30	62.70	202.20	59.80	189.20	59.25	195.20	58.00
C. Organic phosphorus										
Acid-extractable fraction	15.96	16.30	52.70	16.93	53.80	15.95	57.00	17.95	55.90	16.44
Phospholipid fraction	4.00	4.10	3.00	.97	3.60	1.04	5.00	1.57	4.40	1.35
Nucleic acid fraction	20.20	20.80	20.20	6.53	23.40	6.91	21.90	6.80	22.80	6.78
Acid-insoluble residue	12.40	12.70	15.90	5.12	18.00	5.30	18.00	5.63	18.20	5.43
	52.56	53.90	91.80	29.55	98.80	29.20	101.90	31.95	101.30	30.00
D. Total phosphorus recovered										
Acid-insoluble	12.40	12.70	15.90	5.12	18.00	5.30	18.00	5.63	18.20	5.43
Acid-soluble	50.16	51.40	247.00	79.63	256.00	75.75	246.10	77.20	251.10	74.44
Phospholipid	4.00	4.10	3.00	.97	3.60	1.04	5.00	1.57	4.40	1.35
Nucleic acid	20.20	20.80	20.20	6.53	23.40	6.91	21.90	6.80	22.80	6.78
	86.76	89.00	286.10	92.25	301.00	89.00	291.00	91.20	296.50	88.00

*Indoleacetic Acid.

(Data from L. G. Holm.)

complexes as mediators in energy transfer reactions in cellular syntheses, it appears that auxin also must be concerned in these phosphorylation systems. It seems, however, that its role in morphogenesis can hardly be restricted to the regulation of the rate of a single reaction leading to the formation of a single final product, but it must at least include the regulation of the relative rates of coordinated synthetic reactions so as to modify both the quantities and proportions of several products.

The correlative and morphogenetic actions of auxin which are well established in bud inhibition and root formation must be extended to include the formation of buds. For even though the addition of IAA in our experiments results in suppression rather than promotion of bud formation, the marked effect of auxin on cell proliferation together with its properties of high physiological activity and unique mode of transport must be decisive in furnishing new cells, in setting the stage at which these become differentiated into primordia, and in enabling the latter to develop as organized units.

The plan of the present work has been based in part on the early observations indicating that the conditions leading to induction of bud formation are similar to the conditions required for the subsequent growth of the buds. Some results have been obtained which show that separate factors may become limiting in these two processes. Nevertheless, the general conclusion from all the results is that the correlative mechanism which determines the relative growth of organs, and hence the final form of the plant, also functions at the level of cellular differentiation into tissues and primordia.

Keeping in mind the fact that in our present state of ignorance, growth of any kind is far too complex a phenomenon to be interpreted in detail, the formulation of simple points of view on the nature of growth and developmental processes may be useful in guiding further inquiry. For example, the concept of a growth substance regulating the rate of growth of tissues has been extremely fruitful. It has been a prerequisite step in obtaining the evidence, which is now accumulating, to show that substances of many kinds may be preferentially produced or concentrated in certain tissues or organs and that any one of a number of such substances may become a limiting factor for growth. However, recently Sachs' old concept of organ-forming substances has been revived. It has appeared in various new forms (2,5), but to the general effect that a specific substance is required for the formation and/or growth of each

kind of plant organ, that such morphogenetic substances are synthesized only in certain cells, tissues, or organs, and/or that they are produced at specified stages of development. The results we have obtained are in disagreement with such concepts. On the contrary our findings suggest that both organ formation and subsequent development are brought about by quantitative changes in amounts and interactions between nutrients and growth factors which are essential for growth of all cells, so that the pattern of development is determined by the relative supplies, through synthesis, transport, and accumulation of these materials at particular loci. On this basis, the morphogenetic capacities of a given cell or tissue are limited not only by its genetical potentialities for syntheses but more often by its morphological environment, that is, by its particular position in the structurally complex plant body. This concept demands that normal growth of cells must lead to a unified general pattern of development in all plants of comparable genetic constitution, but it permits infinite variation in details.

As a working hypothesis, it lacks the conciseness of concepts invoking the participation of a specific substance for each step in morphogenesis, but it offers equal opportunities for the separation of individual growth factors. In addition, it provides an over-all plan for both physiological and morphological experimentation on correlation phenomena, which, we believe, must control structural organization at all levels in the development of plants.

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The Development of Stems and Leaves

F. W. WENT

Tʜɪs paper is not intended as a general review of the field of leaf growth or stem growth factors other than auxin. It will be an attempt to correlate a limited number of otherwise unrelated facts and experimental evidence, special stress being laid on work carried out in the Kerckhoff Laboratories at the California Institute of Technology in Pasadena.

Leaf Growth.—The problem of plant form is usually considered to lie within the domain of the discipline of morphology. Whereas in its inception this was purely descriptive, during the last half century, and especially since the articles by Sachs on "Stoff und Form" (22), an experimental morphology has been developed too. If we restrict our considerations to leaves we can divide the analysis of their form in two broad groups.

On the one hand the shape of a leaf can be considered as a basic concept, much as the atom is the basic unit for a chemist. The idealistic morphology, of which Troll (30) is a recent exponent, considers form as something absolute which may be modified by external conditions but which essentially can not be broken down into constituent elements.

On the other hand leaf form can be envisaged as being the resultant of all factors contributing to the length, width, and thickness of the organ. Such a point of view does not consider form as a category. Most investigators who have to deal with form—experimental morphologists and geneticists—use this approach, and it is the only possible approach for the physiologist. Modern exponents of this point of view are Huxley (13) and Sinnott (26).

Experimental morphology uses the latter approach, and in explaining the differences obtained as a result of particular treatments the prevailing

physiological concepts are embraced. When the differential effects of plant nutrition became known, especially through the work of Klebs, differences in form were generally attributed to differential nutrition (see 8). Thus the earlier explanations based on specific growth substances (22,3) were more or less abandoned. With the increase in our knowledge of plant hormones, they again assume a central part in morphogenetic considerations.

With the knowledge gained in the last ten years about leaf growth factors it is interesting to develop a unified view of leaf shape, which can account for many of the variations we observe within a single plant, differences between closely related genetic races or effects of external conditions, effects of viruses and other diseases, or teratological phenomena.

As a basis for the following analysis we assume that petiole, vein, mesophyll, and stipule growth are independent of each other. This has been shown experimentally in transplantation experiments with peas, in which these parts on the scion were independently influenced by the stock (36,38). The work of D. Bonner has shown that mesophyll growth is controlled by substances such as adenine which are not primarily concerned with stem and vein growth (4,5). There are certainly many other growth factors for mesophyll (21), but for the following considerations it is sufficient that adenine specifically increases mesophyll development in young radish and pea leaves without affecting vein growth. On the other hand, it is possible to increase vein growth without mesophyll development by applying auxin to leaves (40). In general it seems that petiole and vein growth are influenced by the same factors which increase stem elongation. Thus there is a marked separation in physiological response of stem, petiole, and vein growth on the one hand, and mesophyll growth on the other, a difference based upon a different set of growth factors for each type of tissue. For the sake of convenience in this paper the vein growth factors will be named caulocaline, and the mesophyll growth factors will be summarized under the name of phyllocaline without further reference to their chemical nature. Caulocaline and phyllocaline thus are purely physiological names, that is functional, not chemical.

For the above reasons we should consider leaf form as a synthesis of two separate tendencies which are independently controlled by separate sets of growth factors: a tendency to linear development of veins, and to

surface development of mesophyll. By following these tendencies throughout the angiosperms we find that there are two major leaf types which behave differently upon changes in proportions of caulocaline and phyllocaline. These are the palmate and the pinnate leaves. To the latter type belongs also the parallel-veined leaf. In a pinnate leaf a decrease in the amount of phyllocaline results in a narrowing of the distance between the veins, which thus make a smaller angle with the midrib. This results in a narrower leaf. Yet the veins can retain the same length if the same amount of caulocaline is available. In a palmate leaf, on the other hand, the major palmate veins retain the same angle, but a decrease in phyllocaline results in lobing.

Morphology.—There are a few outstanding examples of differential development of leaves which are mentioned in all morphology text books. As a first case we will discuss the form differences between submerged and floating leaves in certain water plants. If we start with the monocotyledons we see there that leaves of *Sagittaria, Alisma Plantago, A. natans* are all linear if they grow under water, which is the case in the young leaves and in older plants which are grown in streaming water. Under those conditions these species can hardly be recognized one from another, but the first leaves which reach the surface of the water and either float on it or stand up above it develop a pronounced blade by widening of the apical part of the leaf. In successive leaves this widening increases until the typical form is reached, but the plant reverts to its linear type of leaves as soon as it is submerged again. In *Potamogeton natans* and *P. heterophyllus* the same difference between narrow submerged leaves and wide floating leaves occurs. In dicotyledonous plants with palmate leaves the submerged leaves are not linear themselves, but the leaf is divided into linear segments such as in *Cabomba, Limnophila*, and *Batrachium*. In those leaves mesophyll development is practically lacking. The floating leaves, however, are entire and have good mesophyll development so that the whole space between the veins is filled. In both of these cases linear leaves or linear lobes must be attributed to a lack of mesophyll growth factors, whereas the vein growth factors are normally present. Another tempting conclusion would be that in these plants the mesophyll growth factors diffuse out into the water just as they diffuse out of germinating peas if these are submerged (5,38).*

*How general this phenomenon is of leaf reduction in submerged leaves, is in-

In land plants a similar phenomenon may be observed. Palmate leaves often have different degrees of division in the same plant. In general the older leaves are more divided than the younger ones up to a certain age when the division decreases again. In this case there also seems to be a differential between vein growth and mesophyll growth; the larger the quotient vein/mesophyll the deeper incised the leaf will be. Good examples of such a series in one plant are given by Krenke (16, figures 112–120). Another extreme case is *Begonia carolinaefolia* (7). The figures of Krenke (15, figures 17–19) for *Broussonetia papyrifera*, are also good examples of differential growth. Goebel (8, page 37) already gives a scheme explaining various leaf forms by differential development of the various regions of the leaf primordia. Velenovsky (32) also mentions in general: "Es geschieht auch nicht selten dass die Spreite an der Rippe teilweise oder ganz an einer oder an beiden Seiten verschwindet."

In the case of bud scales (8,32), we find nice transition series between typical scales completely lacking in leaf blade and normal leaves. Since the bud scales are considered as transformed stipules rather than leaves, we can suppose that at the time of their development there were different amounts of stipule and leaf growth factors present. This conclusion is strengthened by the observation that under certain conditions organ primordia which normally would have developed into scales can give rise to more or less normal leaves (8, page 428). Such observations are frequently made on the second bud scale of pea seedlings. A quantitative expression of such differences (heteroblastic development) was recently attempted by Ashby (2), who did not present a physiological explanation, but only a graphical or mathematical presentation.

Differential vein-mesophyll development is common in leaves on flowering branches. Often leaf shape changes when the flowering condition is attained. Typical and often quoted examples are the difference in leaf form on flowering and vegetative branches of *Hedera helix* and of *Campanula rotundifolia*. In most other plants the leaf implanted just below a flower stand has reduced mesophyll development.

Light.—The effect of light on growth is complex. In the first place

dicated by the following quotation from Velenovsky: "Die Spaltung des untergetauchten Blattes in lineale Abschnitte ist bei den dikotylen Arten eine verbreitete Erscheinung." J. Velenovsky, *Vergleichende Morphologie der Pflanzen* (Prague, 1905), 413.

light is necessary for the formation of various growth factors and the production of carbohydrates, but in the second place the light also determines the reaction of the tissues to the available growth factors either directly or through activation or destruction of growth factors. Let us first consider peas. When grown in near darkness, pea seedlings develop small etiolated leaves, but when the cotyledons are cut off even this small amount of leaf growth stops. It likewise can be almost completely inhibited by complete darkness. This can be interpreted as meaning that phyllocaline is stored in the cotyledons and is activated in the leaves by light.

In squash a similar behavior can be observed. In darkness seedlings do not produce leaf blades, but with sufficient illumination leaves grow out. Squashes differ from peas in that they require over 100 times as much light to have their phyllocaline activated.

In mature plants the phyllocaline is no longer stored in the cotyledons but comes from the mature leaves where it is formed under the influence of light (9). Actually in recent experiments (14) it could be shown that light is required both on the mature leaves and on the leaf primordia for leaf growth in squash plants; illumination of either one alone is insufficient to produce leaf growth.

These facts can be generalized. Seedlings generally do not expand their leaves when grown in darkness. Many seedlings germinating in a dark forest produce only rudimentary leaves, but their stems are decidedly elongated. Thus climbing plants reach light before they have exhausted their cotyledons in useless leaf growth when they germinate in places where there is not enough light to attain the compensation point.

Helianthus plants produce leaves of different shapes when grown in different degrees of shading. In full light the plants are vigorous and have large heart shaped leaves. In light shade the plants reach approximately the same size, the leaves being only slightly smaller. But with further decreasing light intensity the leaf size decreases much more than the length of the stem. Not only is this true, but the leaf form of the shade plants also is different from that of the sun plants. The leaves of the former are elliptical, and the lateral veins make much sharper angles with the midrib than in the sun leaves. This indicates that mesophyll growth is much more reduced than vein growth in the shade. In the heaviest shade the first leaves are still approximately normal in size; apparently the storage of leaf growth factors in the seed is sufficient to

make the first leaves develop, but the successive leaves become smaller and smaller.

Both the synthesis of the phyllocaline precursor and its transformation into the active material require light. On the other hand the caulocaline complex is relatively independent of light. Auxin production can occur and continue for considerable periods of time in complete darkness, and another factor of the caulocaline complex is formed in the root system in darkness. Therefore we should expect the following effects: shade does not decrease stem elongation but prevents leaf development, and strong light causes expansion of leaves.

Recent work (10) has shown the effect of photoperiod on leaf shape. This is more fully described by Ashby (2).

Nutrition.—Much work has been done on the effect of various nutritional deficiencies on the growth of plants. In many plants it is easy to distinguish between the various inorganic deficiencies since the lack of each element has certain specific effects on the plants. These effects are partly expressed as mottling or other color characteristics of leaves. In certain cases, however, pronounced differences in development are produced also. The latter interest us most in connection with our subject. One of the most typical results of zinc deficiency is the so-called little leaf phenomenon (6). In tomatoes, citrus, and a number of other fruit trees the leaves which develop when the plants are grown with insufficient amounts of zinc have characteristic small and narrow leaves which ultimately will affect the whole growth. This effect on leaf growth may also be produced to some extent by nitrogen deficiency. In *Helianthus* and tomato plants narrow leaves or leaflets are formed when an insufficient amount of nitrogen is present in the culture solution. The effect of boron deficiency is different and is confined especially to the growing part, which ultimately dies. In *Helianthus* again potassium deficiency greatly reduces growth in length, although the leaves are still wide and heart-shaped. Similar differences in the leaves of *Potamogeton* were described by Pearsall and Hanby (20). Thus it becomes evident that the nutrition experiments also give clear indications of differential leaf, vein, and stem growth.

Viruses.—A general analysis of the effects of viruses on plants shows that they can be divided roughly into three groups of symptoms:

1. Mottling of leaves and stems, due to chlorophyll defects, in severe

cases causing necrosis of tissues. In the second place growth defects result which can be traced to deficient photosynthesis.

2. Phloem necrosis, which prevents further translocation of food and growth substances, resulting in stunted growth of all organs, high carbohydrate content of leaves, etc.

3. Developmental deficiencies, in which one or more growth processes are affected. This third group is the most interesting from our standpoint, and within it we can recognize different types.

A. Retarded growth of stem, accompanied in the later stages by increased lateral bud development, leading to witches'-brooms. This is typical for deficient auxin supply. In the first place the lack of auxin prevents normal elongation of the main shoot, and secondly the lateral buds can grow out since they are no longer inhibited by auxin. Similar effects may be observed in dwarf varieties of plants, where dwarfing is due to excessive auxin destruction (31). Thus we are led to assume that such virus effects are due to auxin deficiency caused by the virus, and perhaps also to destruction of auxin (*Prunus virus* 3, *Saccharum virus* 3, and *Delphinium virus* 1 are good examples of this case).

B. Retarded mesophyll growth. The early symptoms of this type of virus are narrow leaves. In severe cases the mesophyll may be practically lacking so that only the midrib remains. This would be a typical case where the virus specifically affects phyllocaline without influencing vein growth. As an example may be quoted *Cucumis virus* 1 on *Spinacia*, tomato, etc. and the shoestring virus of tobacco (*Nicotiana virus* 1).

C. Leaf curling due to retarded vein growth. In a few cases this may be accompanied by unchecked stem growth, but more often stem elongation is inhibited as well. In these cases mesophyll growth is more or less normal so that the whole leaf bulges and is corrugated. Examples are *Rubus virus* 3, *Gossypium virus* 1, and *Nicotiana virus* 10.

Thus if we summarize the effects of viruses on growth phenomena, we are led to assume that vein growth, mesophyll growth, and stem growth are unrelated phenomena, which may be specifically affected

by one or another virus. The effects of the viruses are always growth retardations of any of these processes, suggesting that the viruses act by destroying or inactivating specific growth factors or by decreasing their production. Any combination of these effects may be found in various virus diseases. Doubtless a closer analysis of the virus effects would reveal many more specifically affected growth factors, so that observations in this field may give many clues for the existence of further specific growth factors.

Genetical Evidence.—Practically the same disturbances in leaf form and size which result from virus attack can be found as hereditary malformations. Both in tobacco (deformis, see 12) and in tomato (wiry, see 17) forms with linear leaves are known in which the lack of mesophyll is due to single recessive genes, and which look remarkably like shoe-string-virus diseased plants. Since all these abnormal leaves look much alike, it is logical to assume that all the agents affect the same basic growth process, causing the disappearance of phyllocaline. In other pinnate leaves, such as *Antirrhinum* (23), genes cause narrowing of leaves, whereas in palmate leaves a similar gene gives rise to laciniate forms (for example, *Chelidonium*).

Sirks (27) has investigated the inheritance of leaf size in eight varieties of *Vicia Faba*, measuring both width and length of individual leaflets. He interprets his results by assuming a factor (G) for general leaf growth (with various allelomorphs), which acts in conjunction with additional factors for width (W) and length (B + T). Thus he definitely considers leaf growth as caused by independent factors for growth in width and in length.

Such examples can be augmented by citing the work of de Winton and Haldane (41) on *Primula*, in which 5 or 6 leaf form characteristics were recognized, or the work of Shull (25) on *Capsella Bursa-Pastoris*, or the many investigations on cotton (see, for example, Stephens, 29).

It is interesting to note that mutations which seem to exert their effect by decreasing the amount of special hormones are common to a number of plants. In addition to the phyllocaline-destroying genes could be cited the auxin-destroying genes, which cause dwarfing and often excessive branching.

Morphogenetic Effects of Certain Growth Substances.—Zimmerman (43) has described the effect which many substances with an auxin-like structure have on leaf development. Since then many other chemicals

have been developed which produce similar effects. There is no general correlation between growth-promoting effects and effects on reduction of leaf blade development (see for example, Zimmerman's paper on page 176). The effect of 2,4-dichlorophenoxyacetic acid on tomato or cotton leaves is much like that of shoestring virus or the genes discussed above. Since all these widely different agents cause the same final results, it must be concluded that they all affect the same basic, and probably simple, process, like the phyllocaline-caulocaline balance suggested in the previous paragraphs.

Taxonomic Evidence.—In a large number of genera, species are distinguished by leaf characteristics, and again here narrower or broader leaves or laciniate and hardly incised leaves characterize different species. Edgar Anderson's analysis (1) is an example of how taxonomists and morphologists move hand in hand towards a physiological explanation. He made a comparison between the closely related species *Nicotiana Langsdorffii* and *N. alata* and found them to differ in 11 genetic coefficients. Among these the leaf-vein angle was one, a sharp angle being correlated with narrow leaves and narrow corolla lobes.

In *Sidalcea* the basal leaves are crenate or crenately incised. Most species (*S. malvaeflora*, *S. oregana*) have the upper leaves palmately twice cleft into linear divisions. But in other species the upper leaves are not parted or divided. In *Ranunculus* or *Delphinium* some species are also characterized by leaves which are divided into narrow segments whereas other species have almost entire leaves.

Teratological Evidence.—In any book on teratology a host of examples can be found in which a normally broad-leaved plant bears exceptional narrow leaves, or where palmately divided leaves are found on otherwise entire-leaved plants.*

This enumeration has stressed the differential effects of light, nutrition, viruses, genes, growth substances, normal and abnormal development on caulocaline and phyllocaline production or action. Yet this does not mean that leaf growth is that simple. When we know more about the factors controlling leaf growth we undoubtedly will find that more are involved. In the genetic analysis more than two sets of gene pairs were necessary to account for the observed differences in leaf form. With only two sets of leaf growth factors involved, leaf form would be much less variable. Yet it seems that many of the most common variations in

*M. T. Masters, *Pflanzen-Teratologie* (Leipzig, 1886), 482, 483, 516, 517, 518.

leaf form can be accounted for by the two-factor scheme of phyllocaline-caulocaline in their relative proportions.

Stem Growth.—Very little new evidence has been collected during the last few years concerning the role of factors other than auxin in the elongation of stems. That such other factors exist was assumed as soon as the work with auxin started (33) and was further supported by subsequent experiments (34,35,37). Schneider (24) also found that in addition to auxin and sugars some other factor controlled growth of *Avena* coleoptile sections. In a few experiments Went and D. Bonner (39) were able to replace this factor with pea diffusate or yeast extract, but these experiments could not be repeated consistently. Therefore all our knowledge about caulocaline, as this factor has been named, is circumstantial. In this respect it rather resembles florigen, with which it also has in common the fact that it moves only through continuous vascular connections. When a stem is cut and grafted on another base, growth is resumed only after the graft has "taken" or in other words has new vascular tissue between the graft partners (11). This was also found in the case of the transmission of the flowering stimulus (42).

Synthesis of caulocaline takes place in roots, and in exceptional cases in stems (for example, Asparagus 18). In branches of deciduous plants and in seeds it can be stored in limited amounts, but otherwise stem elongation occurs only when such stems are connected through vascular elements with roots. Therefore stem cuttings of nondeciduous plants do not start to grow until new roots have appeared. Similarly detached buds grown under sterile conditions start to elongate only after roots have been formed on them (21,28). This is even partly true for Asparagus stem tips, whose growth is much speeded up when roots regenerate on them (18).

Summary.—Growth of leaves can be differentiated into vein growth, under the influence of the same factors as stem growth (caulocaline), and mesophyll growth, controlled by different growth factors (phyllocaline) among which adenine has been identified. Differences in leaf form can often be interpreted as being due to differential vein and mesophyll growth. Thus it seems that the form of submerged leaves, of shade leaves, of many virus-infected leaves, of laciniate and other genetically controlled leaf forms is due to lack of mesophyll growth factors. Also in other cases, where for example, virus has caused decreased

vein growth, the leaf shape can be explained by the phyllocaline-caulocaline balance. Hardly any new data concerning caulocaline have been collected. Parallelisms between caulocaline and florigen are pointed out in that both are translocated inside the plant only through intact vascular connections, and both have defied most of the attempts at isolation.

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Growth Substances
in Reproductive Development

Chemical Regulation of Sexual Processes in Fungi

JOHN R. RAPER

I N 1880 Sachs (61) advanced the hypothesis that the differentiation, development, and the proper function of vegetative and sexual organs of plants depend upon the activity of specific chemical substances. Shortly thereafter chemical correlative mechanisms were postulated to explain a number of well-known sexual phenomena in fungi: conjugation between smut spores (13), antheridial production in *Saprolegnia* (10), hyphal fusions (70), and so on. None of these, however, claimed a more definitive basis than deductive speculation.

In the seventy years which have elapsed since the publication of Sachs's work numerous authors have contributed to the greater understanding of the role of chemicals in the regulation of sexual processes in fungi. These contributions fall naturally into two categories dealing respectively with 1) the chance effects of various chemical substances upon the sexual process as measured by the quantitative and/or qualitative effect upon the final product of sexual reproduction, and 2) the effects of specific chemical substances secreted by a plant and playing indispensable roles in the regulation of its own sexual process or of those of another and compatible individual of the same species. The first type of regulation may be considered as a biological accident, whereas the second type of regulation constitutes an integral and necessary part of the mechanism of the plant's vital reproductive process, and the chemical regulators, originating and exerting their physiological role within the plant, may be considered as true hormones.

The first category will be reviewed briefly here while the second will be given more thorough attention with considerable detail of those all too few cases where the broad outlines of the chemical correlative mechanisms have been elucidated.

Extra-specific chemical regulation of sexual processes.—Among the earlier works on the relationship to sexual reproduction of chemical substances, nutrients, or other compounds in the medium, are those dealing with the factors influencing sexuality in the water molds, members of the aquatic order Saprolegniales. The works of Klebs (25,26,27), Kauffman (24), Pieters (42), and Coker (15) established the general pattern of environmental conditions necessary for the production of sexual organs and traced the effects of many variants in the chemical environment of the plant.

The first of a large number of papers, continuing to the present time, on the interspecific effects of metabolites on sexual reproduction appeared in 1903 when Molliard (37) demonstrated that the relative abundance of ascocarps of *Ascobolus* was greatly increased by the presence of certain bacteria. Heald and Pool (23) described a similar stimulation of the sexual process in *Melanospora pampanea* by the metabolic products of certain species of *Fusarium* and *Basisporium*. In the latter case the effective agent was shown to be a complex organic acid. Sartory (62, 63,64) demonstrated the effectiveness of bacteria in association with a yeast and with two species of *Penicillium* in promoting sexual activities in these fungi. Dodge (17,18) observed the beneficial effects of certain bacterial associates on the fruiting of *Ascobolus magnificus* and also pointed out the probable role of the plant's own metabolic activities on the sexual process. The formation of perithecia of *Thielava brasicola* was likewise found by McCormick (36) to be greatly increased by extracts of other fungi. The number and size of perithecia of *Venturia inequalis* were shown by Wilson (71) to be greatly increased by a heat-labile component of the filtration-sterilized brei of *Penicillium* sp.

Robinson (59), in an extensive investigation of growth and sexuality in *Pyronema confluens*, demonstrated the close dependence of sexual reproduction on the chemical nature of the medium and the essential role of light in initiating the immediate conditions for the formation of sexual organs and for the development of ascocarps. Comparable work on a number of species of *Phytophthora* by Gadd (20), Lester-Smith (35), Ashby (4,5,6,7), and Leonian (31,32,33), complicated by uncertainties as to the pattern of sexuality obtaining in the genus, finally resulted in the demonstration of beneficial effects upon the production of oögonia and oöspores by extract of peas and by extracts of numerous unicellular green algae. The influence of C/N ratio upon sexual vigor in *Mucor*

heimalis and *Phycomyces Blakesleeanus* was investigated by Schopfer (65) and Ronsdorf (60) respectively. In *P. Blakesleeanus* phloroglucin completely suppressed sexual activity while histamine greatly augmented the production of zygospores. The only demonstrated instance of a significant effect upon the sexual process of a fungus by a mammalian sex hormone is that described by Plumb and Durrel (43) in which oestrin or theelin was shown to suppress completely the production of gametangia and zygospores in *Rhizopus nigricans*.

The abundance of perithecia of *Melanospora destruens* was shown by Hawker (8,21,22) to be greatly increased by the presence of contaminating colonies of *Botrytis*, *Fusarium*, *Gleosporium*, *Sclerotinia*, and *Penicillium*. Lentil extract also stimulated perithecial formation; this effect was proved to be due to thiamin and biotin, each of which alone would support vegetative growth in a synthetic medium while both were required for sexual activity. Inositol, which was shown to have no effect on *M. destruens*, was shown by Raper (45,47) to increase the intensity of the sexual reaction in matings of *Achlya ambisexualis* in an agar medium.

The requirements for sexual reproduction in *Phycomyces Blakesleeanus* was the subject of an extensive investigation chiefly by Schopfer and Robbins and their associates during the decade 1932–42. Thiamin or its pyrimidine component is required for vegetative growth but other accessory substances were considered necessary for zygospore formation (66,67,68). Such factors were obtainable from numerous natural substrates including malt, oats, potatoes, and agar (52,53,54). The activity in potatoes consisted of two mutually augmentative factors, Z_1 and Z_2, the former adsorbed on Norite, the latter not adsorbed on Norite (56). Factor Z_1 was identified as hypoxanthine (58) and was replaceable by guanine (57), a closely related purine-base compound. The contention that specific accessory substances were required for zygospore formation was disputed by Leonian and Lilly (34) who claimed that sexual sterility resulted from nutritional inadequacies. Zygospores were formed in a synthetic medium containing thiamin, sugar, and only one of a number of amino acids (aspartic acid being the best one). Succinic acid increased the abundance of zygospores while ammonium nitrate completely suppressed their formation; the two effects were partially antagonistic.

The most completely elucidated interspecific stimulation of sexual processes is that of *Zygosaccharomyces* sp. described by Nickerson and

Thimann (40,41). This species in pure culture gave only 8 to 12 per cent conjugation while in cultures contaminated with *Aspergillus niger* the incidence of conjugation was increased to 65 to 75 per cent. This effect could be readily duplicated by filtrates of the culture fluid in which the contaminating *Aspergillus* had grown. The yeast itself was shown to contain the active substance but in low quantity. The incidence of conjugation in slant cultures was shown to be much higher in the thin end of the slant and this could be correlated with the higher percentage of dead cells in this region as shown by staining with Methylene Blue. This points to the possible role of diffusible substances originating from moribund or dead cells in aging cultures in the normal reproductive process of the yeast. The stimulating activity of the filtrate of *A. niger* proved to be organic and to consist of two fractions, an organic acid and a member of the vitamin B complex. Each alone was active but together gave activity greater than the sum of their single activities. The stimulatory effects of these two substances could be duplicated by glutaric acid and riboflavin. Whether these two compounds are identical to the active substances secreted by *A. niger* and contained in the cells of *Zygosaccharomyces* has not been determined.

In Table 1 is presented a summary list of the demonstrated cases of interspecific stimulation of sexual processes and of the few cases where known chemical compounds exert a significant effect on sexual reactions in the fungi.

Intra-specific chemical regulation of sexual processes.—The first demonstration of the initiation and coordination of sexual reproductive processes by diffusible, autosecreted substances was given by Burgeff in 1924 (14) for *Mucor mucedo*. In matings of (+) and (−) strains of this species on an agar medium a restraint area was formed along the line of intermingling of the two mycelia and into this region only a few hyphae penetrated from each mycelium. At the tips of these hyphae corraloid swellings developed before any contact was established and there were produced upon them what appeared to be progametangia. This was considered to be the initial stage in the sexual reaction and since it occurred before contact of (+) and (−) hyphae it indicated the presence and activity of diffusible chemical messengers. To test this hypothesis Burgeff performed the classical experiment of placing a block of agar containing (+) hyphae on the surface of a (−) mycelium growing upon an agar medium with a permeable collodian membrane interposed be-

TABLE I

Extra-Specific Chemical Regulation of Sexual Processes in Fungi

SPECIES AFFECTED	SOURCE	EFFECT	IDENTITY	AUTHORITY
Ascobolus sp.	bacteria?	Apothecia +	?	(37)
Melanospora pampanea	Fusarium spp. Basisporium gallarium	Perithecia +	Organic acid	(23)
Pilobolus sp.	Sordaria longicaudata	Zygospore +	?	(23)
Ascobolus magnificus	bacteria?	Apothecia +	?	(17)
Yeast?	bacteria?	Sporulation +	?	(62)
Penicillium sp.	bacteria?	Perithecia +	?	(63, 64)
Theilavia brasicola	Other fungi?	Perithecia +	?	(36)
Phytophthora cactorum	Peas Green Algae	Oogonia +	?	(34)
Venturia inequalis	Penicillium sp.	Perithecia +	?	(71)
Phycomyces Blakesleeanus	——	Zygospores + Zygospores −	Histamine Phloroglucin	(60)
Rhizopus Nigricans	Mammals	Zygospores −	Oestrin Theelin	(43)
Melanospora destruens	Spp. Fusarium, Botrytis, etc. Lentils	Perithecia + Perithecia +	? Thiamin Biotin	(8, 21, 22)
Achlya ambisexualis	Lentils	Oogonia +	i-inositol	(45, 47)
Phycomyces Blakesleeanus	Potato, Oats, Malt, Agar, etc.	Zygospores +	Z_1, Hypoxanthine (Guanine) Z_2, ?	(57, 58)
Zygosaccharomyces sp.	Aspergillus	Conjugation +	1. Glutaric acid 2. Riboflavin	(40, 41)

tween the two compatible mycelia. Corraloid swellings, the zygophores or sexual organ primordia, formed on the (−) mycelium opposite new growth across the membrane from the agar block containing the (+) hyphae. Thus activation without contact at a distance, or telomorphosis, was proven. Also in a number of cases compatible zygophores on opposite sides of the membrane were seen to grow towards each other; this he termed zygotropism. Thus the initiation of the formation of sexual organs and their correlated development were proved to be the result of hormonal action.

These results were confirmed and in some cases extended by a number of workers on *M. mucedo* and other heterothallic members of the Mucorales. Verkaik (69) claimed that only the (+) strain could be induced to initiate progametangia in membrane matings and that therefore only the (−) strain secreted a stimulatory substance. Ronsdorf (60) and Kohler (28), working with *P. Blakesleeanus* and *M. mucedo* respectively, found that zygophores could be induced on the mycelia of both strains and that at least two substances must be involved; one from each strain acting upon the mycelium of the opposite and compatible strain. The former worker attempted unsuccessfully to extract the active substances from the mycelia of both strains. The activity of histamine in augmenting the sexual reaction intensity in matings of (+) and (−) strains was interpreted as indicating a close chemical relationship between this substance and the sexual hormones secreted by the plants.

Krafczyk (29,30), in experimental work on *Pilobolus crystallinus*, postulated much more extensive activity of sexual hormones than had been claimed by previous workers. The mutual attraction of vegetative hyphae of the two sexual strains before the formation of zygophores, the initiation of sexual organ primordia, the coordinated enlargement of the progametangia while in contact at their tips, and the delimitation of the gametangia by septation of the progametangia he considered to be under hormonal control, that is, hormonal control of the entire sexual process until the time of gametangial differentiation.

In none of the Mucors, however, has sufficient evidence been obtained to postulate either the number of substances involved, the loci of their origins, or their specific activities in correlating the various phases of the sexual reproductive process.

In 1931 a paper by Moreau and Moruzi (38) touched off an interesting controversy. These workers claimed the production of perithecia as the

result of diffusible hormones acting between compatible strains of *Neurospora* in the absence of hyphal contacts. Two compatible strains inoculated in the opposite ends of a U-tube were alleged to produce perithecia although no mycelial growth bridged the connecting tube between them. Extensive efforts to duplicate these results were made by Dodge (19) and Aronescu (2,3) who, by genetical methods, clearly demonstrated that whenever perithecia appeared it was the result of association of nuclei (hence of mycelia) of both compatible strains. Further evidence for hormonal induction of perithecia by Moreau (39) was unconvincing.

The refutation of Moreau's extensive claims, however, by no means indicates that hormones are not involved in the sexual process of *Neurospora* and related forms. Two observations by Backus (9) would indicate that they might well be. The first is that conidia placed on medium or even on a glass surface over which the vegetative mycelium of the compatible strain has grown will not germinate although they retain their ability to fertilize compatible ascogonia. The second, lateral branches arise from the trichogyne only at points normal to the shortest distance to compatible conidia, microconidia, or hyphae, and that such lateral branches grow directly to the fertilizing element.

A case similar to the latter has been described by Zickler (72) in *Bombardia*, in which the tips of the trichogynes are strongly attracted by and grow to masses of microconidia, or spermatia. That this is a chemotropic response was shown by 1) a capillary filled with an extract of spermatia and introduced into the agar in the vicinity of compatible trichogynes caused directional growth of the trichogynes toward and into the tip of the capillary, and 2) an agar block soaked in the filtrate of liberated spermatia had the same directional effect on the growth of trichogyne tips. Boiling had no adverse effect on the activity of the extract and filtrate.

It is only in a few species of the aquatic phycomycetous order Saprolegniales, however, that sufficiently detailed information has been obtained to approach a definition of the over-all hormonal coordinating mechanism of sexual processes in the fungi. As early as 1881 de Bary (10) suggested that a chemical substance secreted by the oögonial initial induced the formation of antheridial hyphae and exerted over them a chemotropic effect. Kauffman (24) induced antheridial production in a species normally lacking the male sexual organs by adjusting the content

of inorganic salts in the medium; he interpreted these results as affecting the conditions under which de Bary's postulated substance would be secreted or effective. Couch (16) suspected specific chemical substances as correlative in the mating of male and female strains of *Dictyuchus monosporus* on the basis of the following observations: 1) oögonial initials and antheridial hyphae were formed on reacting mycelia at locations far removed from the region of actual contact; 2) the directional growth of antheridial hyphae to the oögonial initials; and 3) the marked stimulation, including sexual organ formation, in intergeneric matings with *Thraustotheca primoachlya*. He devised experiments, including the effects of extracts and filtrates, membrane matings, and matings in which certain of the partners were not in actual contact, but the results were in each case negative.

Bishop (11,12) found definite evidence for the activity of diffusible hormones in *Sapromyces Reinschii* of the Leptomitaceae. He demonstrated the production of antheridial hyphae and oögonial initials on male and female mycelia before contact and the induction of greatly increased branching of male hyphae under the influence of the filtrate of a female culture. These effects as well as the directional growth of antheridial hyphae to the exact distal ends of oögonial initials he attributed to diffusible hormones. No attempt, however, was made to define the minimal mechanism necessary to explain these effects.

The hormonal mechanism regulating the sexual process has been more completely worked out by Raper and associates in two heterothallic species of *Achlya*, *A. ambisexualis* and *A. bisexualis*, than elsewhere in the fungi. The various phases of the sexual process up to and including the differentiation of oögonia and the delimitation of oöspheres (eggs) has been shown to be initiated and coordinated by specific diffusible hormones (44). A number of lines of evidence indicated hormones as the coordinating agents: 1) the unvarying sequence of events and the pattern of temporal relationship between the successive stages; 2) the different and characteristic stages of the sexual progression attained in the reciprocal interspecific matings between the two heterothallic species; and 3) the effects of variations in the nutrient content of the medium upon the sexual reaction (45). Conclusive proof of the hormonal mechanism as well as the means of determining the number of hormones involved, the loci of their secretion, and their specific activities has been

furnished by distance reactions (telomorphotic reactions). These reactions include among others initiation of and correlation of organ development before contact of the two sexual strains in matings in water and on agar, matings with permeable membranes interposed between the two partners, and the effects of filtrates (46,48,49).

No less than six distinct hormones are now known to be involved in the coordinative mechanism: four secreted by the male and two by the female. The hormone mechanism as it appears on the basis of information currently available is given in Table 2.*

The entire sexual reaction is initiated by the simultaneous secretion of three substances by the vegetative male and female plants: hormone A by the female, hormone A', an augmenter of the activity of hormone A, and an inhibitor by the male plant. This complex of hormones induces and regulates the formation of antheridial hyphae on the male plant, the number of male sexual organ primordia being a logarithmic function of the concentration of hormone A and a linear function of the concentration of hormone A' (48,49,50). The quantitative production of antheridial hyphae depends also on a number of physical factors including temperature, hydrogen-ion concentration, electrolyte concentration (48), and under certain restricted conditions, light intensity (50). Hormone A is known to be indispensable to the reaction, but whether this is also true of hormone A' has not been determined since the only means of determining the effect of hormone A' is the quantitative reaction of the same plant which secretes it (49).

The antheridial hyphae, beginning shortly after their initiation, secrete hormone B which induces the initiation and controls the development of the oögonial initials, the female sexual organ primordia, on the female plant. The oögonial initials during the period of active growth and until the time of their delimitation secrete at least one hormone, hormone C. If only one hormone is involved at this stage it has two distinct effects: the attraction, that is, the directional growth of the antheridial hyphae along the concentration gradient to the source of the secretion, the oögonial initial; and the delimitation of a short segment of the tip of the antheridial hypha as the male gametangium or antheridium, but only

*Two subsequent papers by the author, *Bot. Gaz.*, 112: 1-24 (1950) and *Proc. Nat. Acad. Sci.* (in press), deal respectively with a newly discovered, ♀-secreted hormone and with the roles of sexual hormones in homothallic species.

TABLE 2

Sexual Hormones in Achlya

MALE
VEGETATIVE PLANT

FEMALE
VEGETATIVE PLANT ⟵—— Malonic, Glutaric Acid

Inhibitor

A'   A

PRODUCTION OF
ANTHERIDIAL HYPHAE

B

PRODUCTION OF
OOGONIAL INITIALS

ATTRACTION OF
ANTHERIDIAL HYPHAE

Thigmotropic response

C

DELIMITATION OF
ANTHERIDIA

D

DELIMITATION OF
OOGONIA

Fertilization

PRODUCTION OF EGGS

MATURATION OF EGGS

after it has become applied to the oögonial initial or some other solid surface. It is quite probable that hormone C is in actuality a complex of two or more distinct substances.

Once the antheridia have become delimited they secrete yet another substance, hormone D, which causes the oögonium to be delimited by the formation of a transverse septum across the base or stalk of the globular initial, and the reorganization of the protoplasmic contents of the oögonium to form a number of uninucleate, spherical gametes (oöspheres or eggs). Again in this stage more than a single hormone may well be involved.

The entire sexual reaction, except for the transfer of nuclei in fertilization, progresses in a normal manner between certain compatible partners separated by a permeable membrane. In other matings (with or without an interposed membrane) the reaction stops at earlier phases corresponding in each particular case with the specific action of one of the several hormones. Interspecific or even intergeneric matings between heterothallic species and various homothallic forms likewise proceed to definite end points in the sexual progression, each characteristic for the specific mating and again to stages corresponding to the specific actions of single hormones. Actually there is considerable evidence that the coordination of the sexual processes in the homothallic forms is similar, excepting hormone specificities, to that of the male plus female mechanism in the heterothallic forms (50).

Isolation and chemical identification has not as yet been possible for any of the hormones involved in the sexual reaction of *Achlya*. The physical and chemical characteristics of hormone A have been worked out in considerable detail, and enormous enrichment of the active compound has been achieved: from 1,440 liters of culture fluid of the female plant of *A. bisexualis*, 37 per cent of the initial activity was concentrated to 0.0002 grams. This material was active in inducing antheridial hyphae on male plants of *A. ambisexualis* in a concentration of less than 10^{-13} (51). The nutritional requirements of and environmental factors affecting hormone A production have been intensely studied but much more information is needed (1,48). The chemical and physical properties of hormone A′ have been subjected only to preliminary investigation. Isolation and/or identification of any of the hormones of *Achlya* will depend on the availability of far larger quantities

of raw filtrates than can be produced with the facilities commonly available in the academic microbiologist's laboratory.

Although there are many instances in which control of sexual reactions in fungi by means of hormones or hormone-like substances have been described or indicated, surprisingly little is actually known of the specific roles of such chemical agents. In all too few cases have studies of such effects been carried beyond the initial observations through the intensive explorations requisite to an understanding of the correlative mechanism. Perhaps the information now available from both fungi and algae, although fragmentary and incomplete, may stimulate work in this biologically interesting, and perhaps in time practically important, field of botanical study.

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The Sexual Substances of Algae

GILBERT M. SMITH

UNION of two gametes to form a zygote is a widespread phenomenon among algae, especially those belonging to the grass green algae (Chlorophyta) and the brown algae (Phaeophyta). Depending upon the alga, there is a union of two motile flagellated gametes, or a union of a small motile gamete and a large immobile nonflagellated gamete, or a union of two nonflagellated gametes one or both of which move in an amoeboid manner.

Sexuality among algae was first recognized by Nathan Pringsheim (17) in 1858 in connection with his study of reproduction in *Oedogonium*, a green alga in which a small motile male gamete (the antherozoid) swims to and unites with a relatively large immobile egg lying within the female sex organ (the oögonium). Within a few years it was suggested that the swimming of antherozoids towards an egg is not a matter of chance but is a chemotactic response to substances secreted by the egg. This assumption was confirmed in the classical studies of Pfeffer (16) where he showed that antherozoids of pteridophytes exhibit a positive chemotactic response to a diffusion gradient of malic acid and certain other organic acids. In this connection it is interesting to note that he reports negative results with gametes of the green algae *Ulothrix* and *Chlamydomonas*.

Subsequent studies on sexual substances of algae have, in the main, been upon heterothallic (dioecious) species where each thallus produces but one kind of gamete. Some of these studies have been upon collections taken from field to laboratory; other studies have been upon clones grown *in vitro* in the laboratory. Irrespective of the source of material, the most satisfactory algae for study of sexuality are those in which both male and female gametes are motile and biflagellate. When one mixes

motile gametes of opposite sex there is an immediate aggregation of the gametes in clumps of ten to a hundred or more. This striking reaction, which takes place in less than five seconds, is so clean-cut that there is never any doubt as to whether there is a positive or a negative sexual response. When there has been a positive clumping reaction the fusion of biflagellate gametes can be demonstrated by killing the swarmers and noting the presence of quadriflagellate zygotes. In rare cases (1,15) the clumping is due to many gametes of one sex swarming about a single gamete of opposite sex. In most cases it is thought, and it is definitely known in certain cases (7,18), that there are numerous gametes of both sexes in a clump.

Up to a certain concentration of gametes the size of the clumps formed after mixing those of opposite sex is correlated with the concentration, but beyond this concentration there is no further increase in number of gametes in a clump. When mixed in optimum concentration the number of gametes in a clump is not the same from species to species and may consist of two, less than half a dozen, ten to twenty, or a hundred or more. For this reason one can distinguish between different intensities of sexual reaction.

The first demonstration of excretion of sexual substances from algal gametes was by Jollos (3) and was by means of differences in intensity of sexual reaction in *Dasycladus*, a heterothallic marine green alga. Since it was impossible to distinguish between male and female gametes on the basis of size or morphology those of opposite sex were arbitrarily designated as plus and minus. When Jollos mixed plus gametes of low intensity with minus gametes of high intensity he obtained a stronger clumping reaction than that from mixing plus and minus gametes of low intensity. He then placed plus gametes of high intensity in a dish, allowed them to swim there for a couple of hours, and then filtered off the water. Plus gametes of low intensity were placed in the filtrate and kept there for a couple of hours. When these plus gametes were mixed with minus gametes of low intensity the clumping reaction was stronger than that with untreated plus gametes. The same change in reaction was induced in minus gametes of low intensity. Jollos interpreted the increased sexual intensity induced in gametes of low intensity as being due to an absorption of sexual substances excreted into the water by gametes of strong intensity. These experiments also show that both male and female gametes excrete sexual substances. Geitler (2) has also shown that

zoogametes of both sexes of the green alga *Tetraspora lubrica* excrete sexual substances. He placed gametes of one sex in water from which gametes of opposite sex had been removed by centrifuging. This was soon followed by a clumping of the introduced gametes, but not by their fusion in pairs.

The most striking of all results with algae immediately after being taken from field to laboratory are those reported by Moewus (13) for *Monostroma*. This heterothallic marine green alga has a blade-like thallus one cell in thickness and one in which each fertile cell produces a number of biflagellate gametes. A zygote formed by union of two biflagellate gametes enlarges greatly and then has a division of its protoplast into 32 haploid quadriflagellate zoospores. Half of the zoospores develop into female thalli and half develop into male thalli. Moewus placed fertile portions of a thallus in a small amount of water, added quartz sand, ground in a mortar, and then filtered. The effect of the sexual substances in the filtrate upon germinating zygotes was studied and found to be the same with filtrates from either plus or minus thalli, but to vary according to dilution of the filtrate. Mature zygotes placed in an undiluted filtrate formed 64 (instead of 32) small biflagellate swarmers when the zygote germinated. These swarmers fused in pairs shortly after liberation. Mature zygotes placed in filtrates diluted 1:2 or 1:4 produced 32 large biflagellate swarmers. When liberated these neither fused in pairs nor reacted to plus or to minus gametes. Mature zygotes placed in a filtrate diluted 1:10 produced 32 large quadriflagellate swarmers which also showed no sexuality.

In another series of experiments mature zygotes were placed in various dilutions of the filtrate until the beginning of cleavage of their contents and then transferred to undiluted filtrate. Zygotes first placed in dilutions of 1:2 or 1:4 and then in undiluted filtrate produced 32 biflagellate swarmers that fused in pairs when liberated. Those first placed in a dilution of 1:10 produced 32 quadriflagellate swarmers that also fused in pairs when liberated. These experiments with extracts obtained from fertile portions of *Monostroma* show that asexual swarmers can absorb sexual substances from the surrounding water and function as gametes. However, there are certain unanswered questions as to the manner in which the sexual substances affect the swarmers. One question is the manner in which a filtrate containing sexual substances extracted from a female plant, or those extracted from a male plant, causes half the

swarmers from a germinating zygote to function as female gametes and half to function as male gametes. Another question is the correlation between concentration of sexual substances in the extract and the number of flagella borne by swarmers from a germinating zygote.

Algae which can be grown *in vitro* are the most satisfactory for study of sexuality because certain of the external factors affecting the formation of sexual substances can be controlled. To be suitable for study in pure culture the alga must be one which 1) grows rapidly in culture; 2) is heterothallic and with both kinds of gametes motile; 3) produces gametes readily; and 4) produces them in abundance. Many algae which can be grown in pure culture fail to meet all of these requirements. For example, *Chlorella* and *Scenedesmus* grow rapidly in culture but never reproduce sexually. On the other hand, such gamete-producing algae as *Ulva*, *Enteromorpha*, and *Cladophora* grow slowly when cultured in the laboratory and do not produce gametes readily. Among the algae meeting all the foregoing requirements are the siphonaceous algae *Protosiphon* and *Botrydium*, and various unicellular Volvocales including *Chlamydomonas* and *Polytoma*.

The nature of sexuality has been studied most extensively in *Chlamydomonas*. When grown in a liquid culture-media this alga is in a motile unicellular state. When grown on a moist substratum, as on agar, *Chlamydomonas* forms a palmella stage in which all cells of the culture are without flagella and lie embedded in a common gelatinous matrix. When palmella cultures are flooded with water or with inorganic nutrient solutions the cells develop flagella within an hour or two, escape from the gelatinous matrix, and swim about in the liquid. Students of sexuality in *Chlamydomonas* have found it far more convenient to culture it in the palmella stage and then induce motility than to obtain motile cells from liquid cultures.

Many, if not all, species of *Chlamydomonas* do not have a division of cell contents to form gametes. Instead, any cell can function as a gamete if it contains sexual substances in sufficient concentration. In palmella cultures of species isolated and grown in the laboratory at Stanford the cells do not contain a sufficient amount of sexual substances until the cultures have attained a certain age, and this age is not the same for all species. At first cell division in these cultures is at a rapid rate, later the rate becomes slower and slower. It is thought that here the sexual substances accumulate at a constant rate, but that in most strains rapidly

dividing cells do not accumulate these substances in sufficient concentration before the amount is halved or quartered by division to form two or four daughter cells.

The most extensive studies on sexual substances of *Chlamydomonas* are those of Moewus on three interfertile species, *C. eugametos*, *C. Braunii*, and *C. dresdenensis*. These three species and mutants derived from them are collectively known as the *eugametos* group. When a palmella culture of any member of the series is flooded with water in light the cells become motile, but if this is done in darkness they do not become motile. Transfer of motile cells from light to darkness is soon followed by a loss of motility. On the other hand, Moewus (8,11) finds that both cells which have become immobile in darkness and palmella cultures flooded in darkness do become motile when various sugars are added to the liquid. Although members of the *eugametos* group can be made motile in darkness by means of sugars the cells do not react sexually when mated with sexually functional cells of opposite sex.

When palmella cultures standing in darkness are flooded with the filtrate from a culture standing in light and containing sexually functional cells the effect is different. Here the cells in darkness become both motile and sexually functional (8). This is due to the fact that the cells in darkness have absorbed both motility-inducing and sexuality-inducing substances excreted by cells swimming in light. However, a filtrate from an illuminated culture will only induce sexuality when applied to a culture of the same sex. That is, a filtrate from a female culture will induce sexuality in a female culture but not a male culture. The reverse is true for filtrates from male cultures.

Having shown that sexual substances of the *eugametos* group are formed only in light, Moewus then proceeded to determine more precisely the conditions under which they are formed and the nature of the substances. He soon found that for both male and female cells the sexual substances are formed only in light from the blue end of the spectrum. Sexual substances are formed in light of 4358 and 4961 Å, but there is no formation of them in light of 5461, 5770, 5791, 5890, or 6430 Å (10).

The effect of light upon sexual substances excreted from motile sexually functional cells was also studied. This was done by obtaining a filtrate containing the sexual substances, exposing the filtrate to light for a specific time, flooding cells in darkness with the illuminated filtrate, and then noting whether or not this procedure induced sexuality in

darkness. When *C. eugametos* is treated in this manner and the filtrate exposed to red, yellow, or green light for several hours there is no loss of the capacity to induce sexuality in darkness. The effect of blue or violet light is different. A filtrate from a male culture exposed to blue or violet light retains, for less than 30 minutes, the ability to induce sexuality in male cells in darkness. When the exposure lasts for more than 30 minutes the filtrate loses the capacity to activate either male or female cells. Filtrates from female cultures are unable to induce sexuality in female cells after exposure to blue or violet light for more than 30 minutes, but exposure of female filtrates to blue or violet light for 75-90 minutes causes a change into substances capable of making male cells function sexually. Filtrates illuminated for more than 90 minutes are unable to induce sexuality in either sex.

Although red light is incapable of causing a formation of sexual substances in *C. eugametos* it is capable of causing a formation of a precursor (P) of them. When Moewus (9) made either male or female cells motile in darkness by means of glucose and then illuminated with red light (5890 Å) there was an excretion of the precursor into the water. When a filtrate from such a culture was exposed to blue light for 20-30 minutes it contained a substance (♀S) capable of making female cells sexually functional. This disappeared after illumination for 30 minutes, but when illumination was continued for 30-40 minutes (a total illumination of 60-70 minutes) the filtrate contained the male sexual substance (♂S). The male substance disappeared after illumination for 10 minutes and neither it nor the female reappeared with further illumination. Illumination for approximately 100 minutes resulted in the formation of an end substance (E). When written as a formula the series of changes may be expressed as follows:

$$P \quad \rightarrow \quad ♀S \quad \rightarrow \quad ♂S \quad \rightarrow \quad E$$
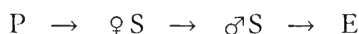
In the change from precursor to end substance there is a gradual decrease in percentage of precursor in the filtrate and a corresponding increase in the percentage of end substance. When the change has progressed to a certain stage the ratio between the two results in the female sexual substance, and a further progressive change in the ratio results in the male substance. Moewus (9) demonstrated this by testing the effect of various combinations of precursor and end substance in inducing sexuality of male and female cells in darkness. For *C. eugametos*

he found that a mixture of three parts precursor and one part end substance made female cells sexually functional, and that one part precursor and three parts end substance made male cells functional. Mixtures in the ratio of 1:1, 2:2, 4:1, and other ratios were without effect on either male or female cells of this species. Later Moewus (10,12) found that the ratios are not the same for all members of the *eugametos* series. According to the species or variety the ratio between precursor and end substance necessary to make female gametes sexually functional may be 95:5, 85:15, 75:25, or 65:35. In this series male cells are, respectively, made functional in mixtures of precursor and end substance in the ratio of 5:95, 15:85, 25:75, and 35:65. For each member of the series the ratio for female cells is the reciprocal of that for male cells: that is, 95:5 and 5:95, 85:15 and 15:85, 75:25 and 25:75, 65:35 and 35:65.

Moewus' studies on behavior of cells in light of various wave lengths and in darkness show that sexual substances present in members of the *eugametos* group are formed in light and disappear shortly after removal from light. Disappearance of sexual substances of the *eugametos* group may be due to a breakdown in the absence of light but this seems improbable because certain other species do not have a loss of sexual substances when transferred to darkness (see p. 325). It seems more probable that sexual substances formed in light by members of the *eugametos* group have the substances diffusing out from the cells almost as rapidly as they are formed. Since there is no new formation of sexual substances after transfer to darkness and outward diffusion of sexual substances continues after this transfer, the cells soon reach a state where they do not contain a sufficient concentration of sexual substances to cause gametic union.

The climax of work on sexual substances of the *eugametos* group came with the demonstration that they are all formed by degradation of the carotinoid pigment, protocrocin (5,6). A molecule of protocrocin breaks down into two molecules of picrocrocin and one of crocin (Table 1). Each of the two molecules of the carotinoid picrocrocin breaks down into a molecule of glucose and a molecule of the carotinoid safranol. The molecule of crocin breaks down into two molecules of the sugar gentiobiose and one molecule of *cis*-crocetin dimethyl ester which, in time, becomes transformed into *trans*-crocetin dimethyl ester. Genetic analysis by Kuhn and Moewus (4) and by Moewus (14) has shown that

TABLE 1

Diagram showing the substances resulting from the degradation of protocrocin

Protocrocin

Picrocrocin → Glucose

Picrocrocin → Safranol

Gentiobiose

Crocin → *cis*-crocetin dimethyl ester → *trans*-crocetin dimethyl ester

Picrocrocin → Gentiobiose

Picrocrocin → Safranol

Glucose
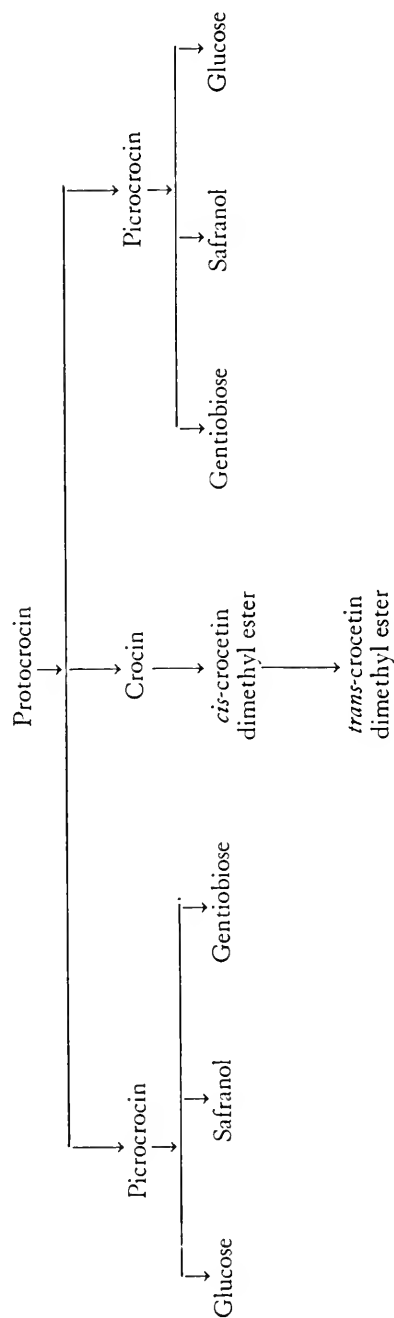
each step in degradation of protocrocin is brought about by a different gene and that at least certain steps in the series are each due to a specific enzyme.

Of the substances produced from protocrocin Moewus (9) identifies the precursor (P) present in a filtrate from cells grown in red light as *cis*-crocetin dimethyl ester, and the end product (E) resulting from illumination of the filtrate with blue light as *trans*-crocetin dimethyl ester. This identification is based upon the fact that a *cis trans* mixture in the same ratio as a P, E mixture also induces sexuality in cells made motile in darkness. When placed in an ascending series according to intensity of sexuality the five sexual types discovered among the *eugametos* group show the following range of *cis trans* differences (Table 2).

TABLE 2

Cis/trans ratios for the five sexual types of the *Chlamydomonas eugamentos* group

	TYPE 1	TYPE 2	TYPE 3	TYPE 4	TYPE 5
cis/trans ratio female cells	65/35	75/25	85/15	95/5	98.2/1.8
cis/trans ratio male cells	35/65	25/75	15/85	5/95	1.8/98.2

For female cells differences in the *cis trans* ratios of the five sexual types approximate very closely the ratios in the Bergmann-Niemann series of (2:1), (2:3), (2x3:1), (2x3²:1), (2x3³:1), (2x3⁴:1), and for male cells they approximate the *trans/cis* ratios (see Table 3).

Since *cis/trans* mixtures induce sexuality in darkness it might be argued that these are the only substances concerned with gametic union. Moewus (12) holds that this is not the case and that there are both gamete-attracting substances (gamones) and sex-determining substances (termones). He holds that *cis trans* mixtures are gamones and that picrocrocin and safranol are termones. The gamone (gynogamone) of a female cell always contains more *cis*- than *trans*-crocetin dimethyl ester, whereas the reverse is true for the gamone (androgamone) of a male cell.

To prove that *cis trans* mixtures (gamones) are not the only substances concerned in sexuality, Moewus (12) took a filtrate from a heterothallic

female culture grown in red light and exposed it to blue light until it
contained the end substance (*trans*-crocetin dimethyl ester). This filtrate
did not induce sexuality in darkness but an addition to it of a *cis/trans*
mixture of the proper proportion did induce sexuality in darkness.
From this he argues that the filtrate contains a sexual substance in
addition to the sexual substance (*cis/trans* mixture) inactivated by blue
light. This argument seems inconclusive when one takes into considera-
tion the fact that *cis/trans* mixtures in distilled water also can induce
sexuality in darkness.

TABLE 3

Table showing the correlation between *cis/trans* ratios of the five sexual types
of the *Chlamydomonas eugametos* group and Bergmann-Niemann ratios

Cis/trans RATIO FEMALE GAMETES OR *trans/cis* RATIO MALE GAMETES	BERGMANN-NIEMANN NUMBER	BERGMANN-NIEMANN RATIO
65/35	2:1	66.7/33.3
75/25	2:3	75/25
85/15	2 x 3:1	85.7/14.3
95/5	2 x 3^2:1	94.7/5.3
98.2/1.8	2 x 3^3:1	98.2/1.8

Moewus obtained more convincing evidence for the presence of
termones in his study of homothallic strains. When taken from darkness
and exposed to light for an hour the cells of a homothallic clone become
motile, aggregate in clumps, and fuse in pairs. Moewus (12) placed
homothallic clones in darkness, flooded them with a solution of picro-
crocin and allowed them to remain in darkness for 20–25 minutes.
These cultures were then taken to light but there was no gametic union
of the cells even after illumination for an hour or more. However, when
the cells had been standing in light for an hour and were tested against
functional male and female gametes they reacted sexually with the
male but not with the female gametes. The picrocrocin makes all cells
in a homothallic culture function as female gametes. Solutions with a
concentration of from 8 x 10^5 to 8 x 10^{11} molecules of picrocrocin per cc.
produce this effect, but those with a concentration greater than 8 x 10^{12}

do not. When treated in similar manner with safranol all cells of a homo-thallic clone functioned as male gametes. This takes place in safranol solutions with 5×10^5 to 5×10^6 molecules per cc. but not in solutions of higher concentration.

From the foregoing experiments Moewus concludes that picrocrocin, or a closely related compound, is the female sex-determining substance (gynotermone) and that safranol is the male sex-determining substance (androtermone). As already noted, picrocrocin is derived from proto-crocin; and safranol, in turn, is derived from picrocrocin, a change brought about by a specific enzyme (14). Female cells of heterothallic species lack this enzyme and so have no conversion of the picrocrocin into safranol. Male cells of heterothallic species produce this enzyme and so have a conversion of all the picrocrocin into safranol. Homothallic species have a conversion of only a part of the picrocrocin into safranol and so have both picrocrocin and safranol within their cells. Cells with more picrocrocin than safranol are female; those with more safranol than picrocrocin are male.

Cultures of members of the *eugametos* group have not been available to others studying the sexuality of *Chlamydomonas* and so there has been no confirmation or extension of the striking results reported for this group by Moewus. However, studies on sexuality in other species of *Chlamydomonas* do afford a certain amount of data for comparison with the results reported for the *eugametos* group.

Plus and minus clones of seven heterothallic strains, none of which is interfertile with any other, and several homothallic strains have been isolated at Stanford University. The heterothallic strains include *C. Reinhardi* Dang., *C. intermedia* Chodat., *C. minutissima* Korshikov, and a strain superficially resembling *C. minutissima* but not interfertile with it. One of the homothallic strains has been identified as *C. Snowiae* Printz.

The behavior of the Stanford strains with respect to light and darkness is quite different from that of the *eugametos* group. When motile sexually functional cells of Stanford strains are taken from light to darkness the cells remain motile and sexually functional for several hours, and in certain cases for a day or two. When palmella cultures of both homothallic and heterothallic Stanford strains are grown in light and then kept in darkness for 24 hours before flooding, the cells become motile within an hour or two after flooding. If plus and minus clones

of heterothallic strains are treated in this manner and mixed in darkness there is a fusion in pairs to form quadriflagellate zygotes.

There are two alternative hypotheses to account for the different behavior in darkness of the Stanford series and of the *eugametos* series: Light is not essential for the formation of motility and sexuality inducing substances in the Stanford series; or light is essential for their formation in the Stanford series but in darkness these substances diffuse out from the cells at a much slower rate than in the *eugametos* series.

A demonstration of the necessity of light in the formation of sexual substances in the Stanford series involves heterotrophic cultivation of them in darkness for many cell generations. Thus far the heterothallic *C. Reinhardi* is the only member of the Stanford series successfully cultured in darkness and only when the carbon source is sodium acetate.

Preliminary experiments with this species showed that when palmella cultures are grown in darkness for several days and then flooded only a small percentage of the cells become motile. If these motile cells are mixed in darkness with sexually functional cells of opposite sex brought from light there is no sexual reaction. On the other hand if cultures grown in darkness are flooded and exposed to white light from a fluorescent lamp all of the cells become motile in about half an hour, but for an hour or so afterward they show no sexual reaction when mixed with functional gametes of opposite sex. After approximately two hours' exposure to light there is a formation of typical clumps sixty or more seconds after mixing with functional gametes of opposite sex. With further illumination the time before the appearance of typical clumps after mixing becomes shorter and shorter, until they are formed within less than five seconds. The number of clumps in a mixture also shows the increased intensity of sexuality as exposure to light continues. In the earliest sexual responses after exposure to light there are only a few clumps after mixing with gametes of opposite sex, but in subsequent tests after further illumination there is a progressive increase in number of clumps. In these experiments the criterion for a development of sexual substances after exposure to light has been a formation of clumps after mixing with functional gametes of opposite sex. This was confirmed by killing the motile cells and finding quadriflagellate zygotes in the mixture.

The foregoing experiments give support to the second of the two hypotheses to account for differences in behavior of the Stanford and the *eugametos* series in darkness. Namely, light is essential for formation

of sexual substances in the Stanford series, but in darkness these substances do diffuse from the cells at a much slower rate.

Unpublished results of studies by H. C. Wendlandt at Stanford show that there is a quantitative relationship between white light from a fluorescent lamp and the development of sexual substances by *C. Reinhardi*. This was done by comparing the time required for the appearance of sexuality in cultures grown in darkness and then exposed to white light of 1, 10, 25, and 50 foot-candles' intensity. The time for development of sexuality is not the same in all cultures exposed to light of any given intensity, but when the average time is taken for experiments in sextuplicate at each intensity the results are consistent (Table 4). There is no appearance of sexuality in cultures illuminated at 1 foot-

TABLE 4

Average number of minutes exposure to white light of increasing intensity required for appearance of sexuality in cultures of plus strains of *Chlamydomonas Reinhardi* grown in darkness when mixed with sexually functional minus cells

Number of clumps in an area of 0.5 sq. mm.	Light intensity in foot-candles			
	1	10	25	50
2 to 4	∞	160	122	112
4 to 8	∞	197	165	136
8 to 20	∞	237	188	160
more than 20	∞	255	225	199

candle, even if this is continued for more than 224 hours. In the other light intensities the time interval for the formation of sexual substances decreases as intensity of illumination is increased, and for each intensity there is a progressive strengthening of the sexual reaction as illumination is continued. Differences in time for sexual substances in plus and minus strains of *C. Reinhardi* are insignificant.

The effect of light of different wave lengths upon the formation of sexual substances has also been studied in various members of the Stanford series. This was done by pouring nutrient agar into Petri dishes, allowing it to gel, inoculating the surface with material from a culture grown in light, and culturing for two weeks in blue light (4357 Å) and in red light (6150–6900 Å). When these palmella cultures were taken to the dark room and flooded the cells became motile and

proved to be sexually functional when tested against the appropriate gametes.

One possible source of error in the foregoing experiments is that the inoculum was from cultures growing in light and that the cells grown in red and in blue light may have contained a small amount of sexual substance because they were but a few cell generations removed from parent cells grown in light. To eliminate this possibility, *C. Reinhardi* was cultured in darkness and material from the culture used as the inoculum for a new culture grown in darkness. Repetition of this for five successive transfers resulted in cultures in which all cells were undoubtedly hundreds of cell generations removed from a parent cell grown in light. Plus and minus cultures of *C. Reinhardi* grown in darkness through five successive transfers were then exposed under combinations of Corning glass filters giving light of the following wave lengths 4200–4600 Å (blue), 5300–5700 Å (green), 5900–6400 Å (orange), and 6200–6800 Å (red). After exposure for 20 hours the cultures were returned to the dark room and flooded. In all cases the cells became motile in darkness and fused to form zygotes when mixed with functional gametes of the opposite sex.

The ability of *C. Reinhardi* to form sexual substances in red, orange, and green light shows that for this species the sexual substances cannot be the combinations of *cis-* and *trans*-crocetin dimethyl ester that Moewus reports for the *eugametos* group.

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Growth-Regulating Substances in Relation to Reproduction of Some Horticultural Plants

A. E. MURNEEK

DURING the past third of a century I have been interested in the physiological aspects of sexual reproduction of the higher plants, and for about a quarter of a century my major research project has been, and still is, on the "Physiology and Reproduction of Horticultural Plants." In this activity quite naturally I have been led recently into studies of certain theoretical aspects of the function of plant hormones and the practical application of so-called synthetic growth regulators with special reference to the production of horticultural crops.

The physiology of sexual reproduction has been till recently, a much neglected field of investigation, as one may judge from inspection of any textbook of plant physiology. The relatively recent discoveries of the striking effects of the photoperiod and temperature, however, have created much interest in this phase of the functional life of plants (93,62). Now the naturally occurring auxins and even more the synthetic growth regulators have come under consideration in flower and fruit development.

Personally, I dislike the widespread use of the term auxin whose original meaning was that of a catalytic substance (hormone) bringing about growth by cell elongation. Recently it has been used to designate not merely one of the three originally discovered auxins, heteroauxin, but practically all synthetic growth regulators employed in experimental work (68). Surely there are many more native hormones in plants than indoleacetic acid. One cannot conceive that as vital and complicated a process as the formation and development of flowers, seeds, and fruit, with their diverse structures and manifold physiological functions,

would involve or be regulated by only one or merely a few auxins.

With your forebearance I shall refer to the naturally occurring form- and growth-controlling substances as hormones and to synthetic chemicals used for this purpose as growth-regulating substances. This despite the fact that, considering the variability in molecular structure, the synthetics per se do not always, and probably rarely, regulate growth and development but most likely only activate or catalyze in some manner a particular native hormone in plants (21,77).

It is my intent to discuss briefly certain salient aspects of sexual reproduction with special emphasis on the possible involvement of the growth regulators. Plants with which we have largely worked and those phases of reproduction of greatest present concern to us perforce will be stressed.

FLOWER INITIATION

Evidence is being accumulated that the initiation of floral primordia is activated by a special flower-forming hormone. It seems to be produced in the leaves, as in photoperiodism, whence it moves to the apical meristems. The formation of this hormone directly in the meristematic regions is not excluded, as with some temperature effects. While no one has yet succeeded in isolating this hormone it has been tentatively named florigen (13). Comparatively recently the idea has been advanced by Gregory (28) that only a florigenic precursor is formed in the leaves under particular environmental conditions and translocated to the meristems where the flower hormone is finally synthesized. Even an anti-florigenic substance, possibly found in leaves on vegetative shoots, has been suggested (48).

After considering most of the experimental evidence indicating the probability of a flower-forming hormone, up to 1938, Cholodny (14) concluded that not a special but the already known native plant growth substance (heteroauxin), under certain conditions of distribution or even absence, most likely causes the transition of terminal meristems from the vegetative to the flowering condition. Though the situation probably is not as simple and direct as this (37,38), considerable evidence has been accumulated recently that certain synthetic growth regulators can initiate or inhibit flower production, depending on the concentration used.

Clark and Kerns as early as 1942 (15), Cooper (16), and subsequently

van Overbeek (70,71) reported that flowering in the pineapple, a rather abnormal parthenocarpic fruit, can be induced with certain synthetic growth regulators. About 50 milliliters of naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D), at a concentration of 5 parts per million, when applied in the whorl of leaves near the apical meristems, resulted in practically all instances in the formation of floral primordia. Too high a concentration of NAA, however, retarded the time of flowering as long as 4 to 8 months (15). Through further tests by van Overbeek this treatment has been put to large scale commercial use. Till a few years ago this was the only instance where flower initiation was controlled by a growth regulator. The following currently published information has further bearing on this point.

By spraying leaves of *Xanthium* plants with indoleacetic acid (IAA) and NAA solutions Bonner and Thurlow (6) were able to suppress initiation of floral primordia during the induction (short-day) period. The same results were secured when only 2 leaves of this plant were submerged nightly during the dark period in a NAA solution of a concentration as low as 1 mg. per liter. In this experiment (6) and a parallel one (7), 2,4-dichloranisole, an antagonist of auxin, counteracted the inhibition of the above chemicals on formation of flowers. Moreover when 2,4-dichloranisole or triiodobenzoic acid were applied to leaves of long-day vegetative plants, flowerlike buds were produced. Leopold and Thimann (46) were able to increase by as much as 35 per cent the number of flowers in barley by applying a weak solution of NAA through the cut surface of leaves. It is of interest to note that relatively high concentrations of the same substance inhibited flower formation. Since both flowering and growth were promoted by relatively low concentrations of NAA, the authors conclude that "these evidences indicate that the growth hormone, auxin, is not necessarily opposed to the functioning of the proposed flowering hormone, but rather influences it in a manner qualitatively similar to its influence on growth."

It would seem desirable to know whether NAA in this instance, while stimulating growth in general, did not retard the multiplication of apical cells in the vegetative meristems thus leading to the formation of reproductive tissues. It has been shown that many synthetic growth regulators may delay or inhibit cell division in the meristem (3,5). In investigations of this kind cytological and chemical studies of terminal meristematic regions during the earliest stages of induction would seem

to be in order (78). Attention should be called here to the reduction of boron deficiency symptoms, necrosis of the apical meristems of plants that have been made reproductive by short photoperiods (91,82). Perhaps it may be considered as circumstantial evidence that, with the onset of reproduction, terminal meristematic growth is not only curtailed but usually inhibited.

FLOWER DEVELOPMENT

Flowers having been initiated, their further development and function would seem to depend upon many internal and external environmental factors. Anyone who has made a study of the growth of flowers is aware of the fact that drastic changes in the environment, such as, for example, marked shift in the photoperiod or temperature, may cause conspicuous changes or even abortion of flower buds, flowers, or parts thereof.

The following stages in flower inception, development, and function may be recognized (55): 1) Terminal meristems or genetically determined loci where the floral hormone is received or synthesized. This is a stage of "ripeness to flower" established physiologically. Commonly there are far more such meristematic points than there are available hormone or other indispensable substances. Many terminal meristems thereby are eliminated early as flower producers. Devernalization and dedifferentiation are well-established phenomena (72,63). 2) Early floral development. If a relatively large number of flowers have started to develop, as seems to be frequently the case, most of them may be eliminated because of competition for supply of building material or specific catalytic substances or hormones. 3) Late floral development. Though reaching anthesis, not all flowers are normal or can function properly (that is, participate successfully in fertilization). Though appearing well developed, they may be abnormal in essential morphological and histological structures. Usually plants produce an enormous number of flowers of which only a fraction form seeds (23,47,65).

There is practically nothing known at present about the function of hormones during various stages of flower development. Though their multifarious forms and structures are determined genetically, most likely hormones of various kinds participate in the production of specific floral organs and tissues.

Gametogenesis and Fertilization

Several years ago in work on problems of the relation of nutrition to reproduction of the tomato, it was found that as a result of fertilization (gametic union) and formation of zygotes not only tissues accessory to the embryo and structures immediately subtending the flower (42,35) are stimulated in development, but that metabolism throughout the plant is accelerated to some extent. This was evidenced by increased absorption of soil nutrients, and assimilation of carbon dioxide (51). It has been confirmed with several other plants (56,94,19,4).

Subsequently two phases of the observed stimulation during the general period of flowering and fruit setting were recognized, one occurring about the time of synapsis and the other, a more important one, during syngamy (54,55,52,57). Stimulation at synapsis, postulated from general observation and gross analyses of the effects, was later fully established by a former student and colleague of mine (95). It appears to be initiated within the partly developed flower buds concomitant with chromosome conjugation in the micro- and macrospore mother cells. In the staminate corn flowers the stimulation reaches a peak about 7–12 days after synapsis. The second stimulation, originating from gametic union in the embryo sac, about the time of fertilization, attained a maximal effect in corn some 15 days after pollination (95).

Undoubtedly responsibility for the two periodic stimulations of growth associated with sexual reproduction lies with a hormone or hormones. Evidence, though not fully conclusive, indicates that it is not due to an increased production of the already well-known heteroauxin. From 100 kilograms of 15-day old corn kernels furnished by us, Haagen-Smit *et al.* (34) extracted all the free *Avena*-testable hormone, of which only 9 per cent was 3-indoleacetic acid. What is the rest? Contrary to the effects of indoleacetic acid, the crude extracts of immature corn kernels, applied in lanolin paste, were highly active in the production of parthenocarpic tomatoes and peppers. Moreover, Stanley McLane, working in our laboratory, found that in the culture of excised immature corn embryos (10–20 days after pollination) the addition of 3-indoleacetic acid, comparable in concentration to that found in extracts from 15-day old corn, inhibited growth, while a water extract of corn, without the natural indoleacetic acid removed, doubled growth of embryos.

Shall we call these unknown hormones synapsin and syngamin re-

spectively? Wittwer (95) found that the growth activity in extracts from corn pollen and immature grains is not destroyed by prolonged heating. According to McLane, syngamin seems to be insoluble in ether at pH 5.4, passes through a collodion membrane, and can be removed in part by treating corn extracts with activated charcoal.

FRUIT SETTING

The present evidence seems to point to the following sequences in the function of hormones in relation to fruit setting. Pollen germinating on the stigma either produces a hormone (32) or through secretion of an enzyme liberates a hormone from inactive combinations in the style and ovary (98,50). The male gametophyte, therefore, has at least two functions, fertilization of the egg and causing the ovary to grow. The latter action seems to be closely associated with prevention of abscission of the young fruit.

Van Overbeek (68) believes that not only the carpels but also the ovules are controlled in their initial growth by a hormone originating in the microgametophyte. By injecting NAA or indolebutyric acid (IB) into the ovaries of *Melandrium* and *Datura* not only parthenocarpic fruit were secured but they contained enlarged ovules with seed coats although no true embryos (66). He suggests that, with some plants at least, even division of the polar nucleus and the egg cell, before fertilization and triple fusion, may be brought about by the presence of pollen tubes in the style. Gustafson (32) has shown that pollen extracts when applied to pistils may cause them to develop into parthenocarpic fruit, while Laibach (45) and Thimann (84) found that pollen contained hormones. However, the greatest hormone effect on the fruit absciss layer probably comes after fertilization from the ovules (1,50), since the hormone content of ovules and developing seeds is exceedingly high (31).

The disclosures by these studies, but probably even more the discovery by Gustafson (30) that several synthetic growth regulators will induce fruit development without pollination, and by Gardner and Cooper (28) that premature dropping of apples can be retarded by spraying with a dilute solution of NAA, have been of considerable value in horticulture. Moreover, it has created much interest leading to further experimental work on the use of growth regulators in horticultural practice.

Through the tests by Zimmerman and Hitchcock (99) about 30

organic chemicals so far have been found effective in fruit setting. Of these the naphthoxy and substituted phenoxy acids are especially potent for this purpose. Many-seeded fruit evidently respond more readily to stimulation in development by synthetic growth regulators than do fruit containing a single seed. Desirable concentrations and the time and mode of application have been studied extensively, particularly with plants with which the greatest success has been secured so far.

Tomatoes.—When tomatoes are grown in the greenhouse during cloudy weather and/or subnormal temperature, or outdoors when the nights are relatively cold, the number and size of the fruit can be increased by the use of IB, NAA, and 4-chlorophenoxyacetic (ClPA) acid, some of their homologues, and a number of other synthetic growth regulators (41,58,61,97). The flowers are usually sprayed when in full bloom or, preferably, after pollination and fertilization have occurred. Pollen tube growth and fertilization, as noted before, in their aggregate effect on growth of the ovary supplement the effects of synthetic growth regulators on carpel development. There does not seem to be any appreciable commercial value although there is a great deal of talk about the production of seedless tomatoes.

Tomato flowers are receptive to stimulation of this kind several days after pollination. If whole plant spraying is practiced, which has been shown to be even more effective than flower-cluster spraying (58, 61), the spray must be so directed as to cover only that portion of the plant containing mostly open flowers and young fruit. It has been observed (58,61) and pointed out by Roberts and Struckmeyer (73) that spraying tomato buds with growth regulators five or more days before pollination gave a poor set and fruit of relatively small size.

Hemphill (40) has recently completed a thorough investigation on the time of application of three synthetic growth regulators at the concentrations most desirable for increase of the tomato crop. When applied eight days before anthesis pollen ceased to develop and collapsed in the anthers, while spraying four days before full bloom often caused premature pollen germination. The ovules were also detrimentally affected when a growth regulator was applied as early as the bud stage. They either ceased to develop and the embryo sacs disintegrated, or those that did grow were greatly retarded in development. Seed formation was usually prevented. These results are in considerable agreement with those obtained by Britten (10), who sprayed NAA on developing

maize caryopses. What is equally important, they throw considerable light on the results observed when NAA is used for the reduction of fruit set of the apple and some other fruit crops.

Green beans.—Abnormally hot weather at the time when string beans are setting fruit may reduce the yield of pods considerably. This can be overcome to an appreciable extent by either spraying or dusting the plants with a desirable growth regulator. It may be combined with a necessary insecticide. Maturity of the pods, as judged by size is also increased by the treatment (59,96,26). The beneficial effect apparently is brought about by stimulation in growth of the carpels (pods) and an increase in chlorophyll content of the leaves. Probably the abscission layer between fruit and pedicel is also strengthened. The seed number in some cases may be reduced but, where the proper concentration is employed, it is usually about the same.

Fruit Thinning

Although we have tried repeatedly to increase the set of stone and pome fruits by the use of synthetic growth regulators, the results have invariably been negative. Instead of an increase, there has commonly been a reduction. This response, too, has been found under certain circumstances to have practical value as will be seen forthwith.

Burkholder and McCown (12) reported that reduction in the crop of apples, when the set is excessive, may be brought about by spraying the trees in full bloom with NAA. This has been confirmed by others (18,75,79), some of whom found that exact timing of the application, as on the first day of full bloom, is not required.

During the past four years we have conducted extensive field investigations on thinning apples and peaches with NAA, with special reference to the time of application and concentration to be used (60,64). The results, still largely unreported, show that this growth regulator is an efficient substance for reduction of the number of fruits on an apple or peach tree. For apples, the best time of application seems to be one to two weeks after full bloom. The concentration of the spray material to be used is 10–30 ppm. depending on the variety and whether it is an annual or biennial bearer. Varieties that are difficult to thin chemically may require two applications of the spray. Peaches may be thinned successfully with NAA, applied about one month after bloom, at a concentration of 30–40 ppm. for the light setting varieties and 40–60 ppm. for

the heavy setters. By using still higher concentrations it is possible to remove the apple crop completely if that be required. This rather late thinning by means of a spray is more desirable than spraying in full bloom at which time it is difficult, often impossible, to tell what the crop is apt to be.

Here then we have another illustration that the same growth regulating substance may have opposite effects depending on the concentration used. At a relatively weak concentration NAA may initiate flower production, foster the formation and growth of fruit and prevent fruit abscission, while at higher concentrations it can stop fruit development and cause their drop.

How can NAA accomplish these rather striking results in fruit thinning without harming the tree or the remaining fruit? Some light may be shed on this question by considering the natural sequences in embryo, endosperm, and fruit development of the peach and apple.

Embryo Development and Fruit Growth

The peach and other stone fruits require the presence of an embryo for their growth. The same is true of the apple and other pome fruit, which usually contain several seeds. Parthenocarpy is a rare phenomenon with the genera *Prunus* and *Malus*. Tukey (85) found that the peach fruit develops in three stages: 1) There is a rapid increase of the pericarp, including nucellus and integuments, for about forty to fifty days after full bloom. During this stage growth of the embryo does not parallel that of the pericarp. It remains embryonic, in a kind of arrested development. Note should be taken of the fact that development of the endosperm usually precedes that of the embryo (27). 2) During stage two the embryo grows rapidly to a maximal size. The duration is five to forty days depending on the variety, that is, how early the fruit ripens. Increase of the pericarp is at a relatively slow rate. 3) The pericarp or fruit increases rapidly in size to the time of fruit ripening. In very early varieties stage three is initiated while the embryo is in a period of rapid growth, with the result that it fails to reach full size (aborts), the nucellus and integuments collapse, the fruit ripens rapidly and drops from the tree. This cyclic type of growth seems to be characteristic of other cultivated drupe fruit.

Tukey (87) artificially destroyed embryos in the peach at various periods. Killing in stage two of development resulted in an abrupt check

of fruit growth and prompt abscission. When the embryo was destroyed between stages two and three the fruit failed to reach full size and ripened quickly. Destruction of the embryo in stage three was followed by increased fruit growth and somewhat earlier ripening. From these and other studies (88) it is quite evident that the embryo has a definite bearing on fruit formation. Naturally occurring hormones undoubtedly play a role in this relationship. Most probably they regulate the metabolism and nutrition not only of the embryo but also of the fruit. Much evidence that hormones are important factors in the development of young fruit comes from the successful use of various synthetic growth regulators in production of parthenocarpy (32). Moreover, it has been found in several instances that seeds of developing fruit are rich in hormone content (22,36).

The situation in the apple as regards the relative period and rate of development of the embryo, endosperm, nucellus, and integuments is similar to that of a drupe fruit (24,80,76). But since the bulk of the apple is made up of accessory tissues (torus), it does not show cyclic stages of growth (89). A fairly good correlation, however, exists between seed (embryo) number and size of apples during early stages of their development (39). Later on, before ripening, this relationship seems to vary considerably depending primarily on the size of the crop and the available food supply (74). Seeds do, of course, have a local effect on growth of the pericarp (86) and torus (74).

By means of artificial culture of embryos of various ages, isolated from their natural environments and associated tissues, information has accumulated as to their nutrient requirements (74,67,69). In most instances it has been found that in addition to the necessary inorganic substances and certain organic nutrilites, an embryo factor is indispensable. This has been discovered in yeast (44, 92, 43), tomato juice (43), malt extracts, and a number of other plant-tissue extracts and substances. Older embryos, being autotrophic, do not seem to require this factor. Van Overbeek (67,69) has successfully used a liquid endosperm, coconut milk, for the culture of young *Datura* and other embryos. At least two important factors probably are present in coconut milk: a thermolabile one, which causes embryos to grow rapidly, and a heat stable factor that inhibits root development of the embryo.

Embryo culture is of considerable aid in obtaining plants from certain self- and cross-sterile matings and in the production of seedlings from

aborted embryos. Eyster (25) has reported that when flowers were sprayed immediately before or shortly after pollination with a weak solution of naphthaleneacetamide, highly inbred self-sterile plants of several species produced viable seeds.

Endosperm and Its Role

In detailed studies of embryo, seed, and fruit development considerable emphasis has recently been placed on the endosperm (9). This 3n intercallary tissue between the new and old sporophyte provides the medium suitable for the growth of young embryos. Usually its development precedes that of the embryo. A failure in the necessary nuclear division or function of the endosperm as a rule results in failure of embryo growth. The endosperm increases rapidly during the early stages of seed development and is digested and absorbed by the growing embryo. Its role seems to be entirely nutritive. There is usually little or no endosperm when the seed is mature, excepting where it has assumed the secondary function of a storage organ.

The important activity of the endosperm in nourishment and development of the embryo of angiosperms has been summarized by Brink and Cooper (1940) which may be paraphrased as follows: Since the female gametophyte is exceedingly small in size and no endosperm is formed till after fertilization, the ovule contains little or no reserve food. The new sporophyte would be thwarted in development were it not for the fact that fertilization initiates not only growth of the endosperm but stimulates to active expansion the adjoining maternal tissues (pollination as noted before, does it also). Originating as uninucleate structures the embryo and endosperm must compete for food supply with the adjoining well-established tissues. Success of the young seed would seem to depend primarily on the endosperm as a nutritive agent of the embryo to assist it in establishing its dominant position in the ovule and ovary. Double fertilization appears to be the method conferring upon endosperm the necessary power through physiological advantages of the hybrid condition. If during early development the endosperm fails to remain dominant the surrounding nucellus or integuments may outgrow it withholding or diverting the food supply from the embryo. This results in starvation of the embryo and collapse of the seed—the so-called somatoplastic sterility (17).

Should one consider farfetched the suggestion that the synergids,

and possibly even the so-called "X-bodies" that flank the egg before and immediately after fertilization, may have a similar function to endosperm but more specifically in nutrition of the egg cell?

Doubtless in the endosperm-embryo relationship hormones have an important function. Voss (90) observed that isolated embryos of corn require for their development a naturally occurring hormone, which could not be replaced by indoleacetic acid. During germination a hormone from endosperm evidently was translocated into the embryo. This has been verified more recently by Guttenberg and Lehle-Joerges (33) who detected in all parts of the embryo a special hormone that originated in the endosperm.

Fruit Thinning Again

Now to turn once more to fruit thinning by means of a growth regulator. In this connection we must consider the state of development of the seed at the time the spray is applied and the possible effect of NAA on the embryo and endosperm. According to Tukey (85) and Harrold (36) microscopic embryos and normal endosperm are present in the peach about the approximate time the growth regulator is applied for thinning purposes. Five- to ten-celled embryos and a many-celled endosperm are present in the embryo sac of the apple up to two weeks after fertilization.

In both stone and pome fruit (peach and apple), with which we are concerned in this discussion, endosperm development after fertilization and triple fusion precedes the division of the zygote nucleus (36,11). It is most important to note here that Bryant (11) observed that, depending on the kind of pollen used in fertilization, the endosperm nuclei seem to vary considerably in number during this early stage of seed development. Growth of the endosperm usually lagged under self-pollination and when a less efficient pollen was used. This reminds one of the differences in endosperm production, as described by Cooper and Brink (17) in distant crosses of *Nicotiana*, *Petunia*, and other species, wherein, however, the endosperm nuclei divided most rapidly after self-fertilization and more slowly in hybridization, resulting in collapse of the young seeds. This may have a considerable bearing on the differential abscission of fruit as a result of spraying with NAA.

That the apple embryo and endosperm when microscopic may be sensitive is indicated by additional circumstantial evidence. As a result

of an extensive survey of fruit setting of the Delicious apple, Gardner *et al.* (29) reached the conclusion that during the critical period of one to two weeks after full bloom the young fruit apparently is very sensitive and may abort easily if the environment, primarily temperature and sunlight, is not favorable. Of even greater interest is the contention that certain fungicides, used for scab control at this early period, may also have a profound effect on fruit set of this variety.

Luckwell (49) has made a seasonal study of the hormone content of apple seeds in relation to endosperm development. The concentrated ether extracts from whole seeds and separately from embryos and endosperm-nucellar tissues (older seeds) were tested for their potency to produce parthenocarpic tomato fruit. The hormone concentration was about 18 times as high in endosperm-nucellar tissues as in the embryo. And since the nucellus constituted only a minor fraction of the sample, the author thinks that the hormone came chiefly from the endosperm. During the period of the usual postfertilization abscission of apples (53,20) the hormone content was of a relatively low concentration. Luckwell expresses the view that the hormone found in endosperm seems to prevent fruit abscission. Does it mean that a hormone released by endosperm, in preventing abscission, would thereby help to maintain or increase the food supply to the seeds and fruit?

Naphthaleneacetic acid, at the relatively high concentration used for apple and peach thinning, most likely disturbed the endosperm and possibly also the embryo in their physiological relationships during the critical period of early seed development. This effect may be a direct one or, less likely, through stimulation of excessive growth of the pericarp. Studies by Swanson *et al.* (83) on ovule abortion in *Tradescantia*, as disturbed by a 2,4-D spray, suggested that the younger the ovule the more harmful was the spray and that endosperm was inhibited in relatively mature seeds. Ovule collapse followed disintegration of the endosperm and the chalaza. Still later the nucellus and integuments collapsed.

Preliminary observations by us of apple fruit thinned by means of NAA indicated invariably an effect on the seed, endosperm, and nucellus, which had collapsed. This probably was the cause of abscission of large numbers of the young fruit. Very probably the specimens that abscissed were most sensitive to the treatment due to the presence of fewer seeds or seeds with less developed endosperm because of partial pollination or pollination with less compatible pollen. Fruit borne on spurs

in relatively weak positions as regards food supply may also be more sensitive in this respect (39).

When still higher concentrations of NAA are employed during advanced development of the apple fruit (20 to 50 days after bloom) the seed collapsed, fruit growth either ceased completely or was greatly retarded, but abscission did not always follow. As yet we have not been able to conduct a detailed study of the histological changes in fruit as a result of NAA application. Whatever the mechanism responsible for thinning may turn out to be, this synthetic growth regulator (NAA) has given us a powerful tool to control the size of the crop of certain fruit.

CONTROL OF PREHARVEST DROP OF FRUIT

Mention should be made, briefly at least, of the well-established practice of using a weak solution of NAA to retard the preharvest drop of apples, pears, and a few other fruits (28). The effect evidently expressed itself through a delay of the natural changes in the abscission layer between the fruit and its pedicel coincident with ripening, which lasts, from a NAA spray or dust, for about 10 days.

Batjer and Thompson (2) have demonstrated that 2,4-D is even more active in its lasting effects for apple drop prevention, but for some unknown reason it seems to work mainly on the Winesap group of apples. Stewart *et al.* (81) have discovered that 2,4-D can prevent also the abscission of citrus fruit.

Our tests (63) point to the fact that 2-methyl-4-chlorophenoxyacetic acid is almost as potent as 2,4-D for length of delay of the preharvest drop of apples and that 4-chlorophenoxyacetic acid stands in a position between the above two chemicals and naphthaleneacetic acid.

CONCLUSION

This somewhat sketchy paper should serve as a brief summary of the present status of studies on the function of hormones in sexual reproduction of some crop plants. Special emphasis has been placed in this discussion on the use of synthetic growth regulators in connection with certain cultural practices of horticultural plants. Objections have been raised to the widespread employment of the term auxin in designating both naturally occurring hormones and synthetic substances used both experimentally and in crop production as growth regulators.

From flower initiation to delay of fruit abscission during the preharvest

period, growth regulators have been found effective tools in the control of development of certain tissues and organs. Their practical application, in several instances, has outrun our present scientific knowledge. As a result of further experimental work additional and even greater usefulness undoubtedly will be found for these catalytic substances in seed and fruit production. Progress in this field certainly would be hastened if more information were available on the detailed physiology of plant reproduction, with particular reference to the crucial phases.

Heretofore the main emphasis in analyses of sexual reproduction has been placed on the embryo and mechanisms leading to its formation and growth. Attention is now being called to the importance of the endosperm and associated tissues in seed and fruit development.

Information has been presented which shows how the same synthetic growth regulator (naphthaleneacetic acid) may have quite opposite effects in sexual reproduction, depending on the concentration used and the time of application especially in relation to seed and fruit development. A relatively weak aqueous solution may initiate flowers, promote fruit set, and retard fruit abscission in some plants, while one of higher concentration will inhibit or delay flower induction, curtail or prevent seed development, stop or retard fruit growth, and cause abscission.

Though probably of considerable importance in the functional life of plants, only a beginning has been made in studies of the over-all effects on metabolism of certain phases of reproduction, with reference to absorption of soil nutrients, carbohydrate assimilation, and other major plant activities. Hormones of various kinds, undoubtedly, have roles here just as in detailed tissue growth and development.

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The Induction of Flowering with a Plant Extract

R. H. ROBERTS

IT IS agreed by workers who have studied the physiology of blossoming, especially as related to photoperiodism, that plants contain a chemical substance which induces flowering (3). This concept is based particularly upon grafting experiments in which a plant in flower or a leaf from a flowering plant will induce a nonflowering plant to blossom when the two are grafted together under the proper experimental conditions. No report of a successful extraction of the blossom-inducing chemical has been seen although the name florigen has been proposed for it by Cajlahjan (1).

Several years ago Struckmeyer (4) reported that there is reduced cambial activity and increased maturation of tissues at the time sexual reproduction is initiated. Thus it appears that the blossom-inducing substance causes maturation of tissues in contrast to the proliferation of cells produced by the numerous growth substances (5), some of which are used as weed killers (2). Since florigen appears to have a physiological effect opposite to the so-called hormones, it was presumed that its extraction should require an unlike procedure, or at least an unusual solvent.

In 1946 a solvent was found which gave promise of being useful in obtaining florigen. The first successful extraction and subsequent blossom induction of the short-day plant cocklebur (*Xanthium echinatum*) with its extract was accomplished in December of that year. The fifty-second

plant to be treated produced about the same degree of blossoming as occurs on plants after exposure to one or two long dark periods (short days). Subsequent trials with crude extract were 8 to 10 per cent successful. In January, 1948, small, colorless, isotropic granular particles were obtained from the extract. When these were dissolved in fresh solvent and applied to vegetative plants of cocklebur in a long-day environment in March, all of the 15 plants which were treated formed staminate blossoms comparable in development with those on plants which had been given one or two short photoperiods (Fig. 1). The macroscopic blossom buds appeared after five to eight weeks. This amount of time is typical of the period usually needed to produce blossoming from the stimulus of a single long dark period. Seven of the 15 plants bore pistillate blossoms similar to those on plants which have received two short photoperiods.

The extract was effective at as high a dilution as one part to ten thousand. The effect from the solution was also additive to photoperiod. Four plants given one short photoperiod and also sprayed with the solution made a greater blossom development than those with only one short photoperiod. A total of 43 cocklebur plants have been induced to flower by treating them with plant extract.

Other effects from applying the plant extract were to reduce cambial activity and to induce maturation of tissues comparable to that resulting from photoperiod treatments which bring about a like degree of blossoming. The extract also inhibits callus formation in wounded areas of stems. It has not yet been determined if a substance of a like physiological activity would have a similar effect upon some animal tumors.

The following procedure is used to obtain florigen particles. Soak a small sample of fresh or frozen leaves taken from plants in flower in the least practicable amount of a highly refined, odorless insecticide base such as Shell Dispersol, for an hour or longer. This solvent is a non-aromatic oil fraction recovered from kerosene and having an IBP of $387°$ F. and an FBP of $485°$ F. Squeeze out the solvent, remove and discard the aqueous phase if any is present, and filter. A yellowish pigment which seems not to interfere with florigen extraction or activity is present in the extract from most species. Deep freezing at $0°$ F. or below is used to initiate separation of the particles. This progresses slowly for several hours or even days. The particles in extracts from some plants, for example, white sweet clover or sweet corn, dissolve at room

Figure 1. Tips of cocklebur plants with older leaves removed. *A*, Non-flowering plant. *B*, Fruiting plant (induced by short days). *C*, Partial fruiting induced by one long dark period. *D*, Nearly normal fruiting induced by plant extract in addition to one long dark period. *E*, Partial fruiting from two long dark periods. *F*, Partial fruiting following treatment with plant extract.

temperatures. The florigen particles are recovered by filtering or decanting, and evaporating the remaining solvent at a temperature below 60° C.

The particles are highly insoluble in such solvents as water, alcohol, acetone, xylol, benzol, ether, ethylacetate, petroleum ether, ethanolamine, polyethylene glycol, and dioxane. This may be an explanation of why attempts by numerous workers to obtain florigen have been unsuccessful. The particles are poorly soluble in Dispersol. Consequently, repeated extractions of the leaves yield added amounts of particles.

No particles have been obtained from extracts of nonflowering plants such as those growing in a photoperiod unfavorable to flowering or from those plants which are not known to flower, as for example, some varieties of sweet potato. Particles of a like physical appearance have been obtained from 23 species of dicotyledons including *Cuscuta* (Dodder) and *Monotropa* (Indian Pipe), and from six monocotyledons. All flowering plants chosen for extraction have yielded particles. Tests are under way to determine if these will have interspecific effects on the induction of blossoming. Preliminary experiments directed towards characterization of the extracted particles are also under way.

A solution of the florigen particles in Dispersol can be applied to plants being used in tests of flower induction by wetting the foliage with it. Plants for a preliminary test of induction should be selected from those with a systemic flowering habit, at least until more is known of the factors determining the induction of plants which flower only terminally. (Plants of the latter type, as Klondyke Cosmos, do not become induced to blossom by grafting.) A plant should not be expected to respond beyond its genetical potentialities.

The limited action of the extract in inducing blossoming of cocklebur comparable to that from only one or two long dark periods may indicate that the present material may be a precursor rather than the actual florigen.

For several months after September, 1948, the induction studies were interrupted by the accidental introduction of a volatile ester of 2,4-dichlorophenoxyacetic acid into the greenhouses. In March, 1949, 16 plants of 19 treated with extract became partially induced, again to about the same degree as plants given one or two long nights. In all 131 plants have been partially induced with plant extract.

Interest has shifted for the present from the induction of blossoming to the nature of the extracts which have been obtained. By varying

extraction conditions six crystalline substances have been secured. These are being termed florigens as they have been extracted from flowering plants and are not obtained from nonflowering plants. Partial characterization under the direction of Dr. Ben Aycock of the department of Organic Chemistry of the University of Wisconsin indicates that they are mineral salts of fatty acids. Poor solubility makes identification and the determination of physiological activity slow, but observations to date indicate that information, which may answer the question of the nature of the mechanism of photoperiodism, will be obtained from a knowledge of the chemical nature of the extracts now available.

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Fruit Development as Influenced by

Growth Hormones

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THE production of fruits is ordinarily associated with pollination and fertilization, and it is almost axiomatic that if there is no pollination there is no fruit setting. In many parts of the country orchardists, to insure fruit setting, have gone as far as to distribute beehives in their orchards during the blossoming period. In spite of this common relationship between fruit setting and pollination and fertilization we find that there are numerous instances in which fruits are produced without fertilization, and not even pollination is necessary in some cases (10). These fruits are seedless either because of lack of fertilization or because the young embryos aborted leaving only tiny nutlets as a reminder of fertilization. Some of our so-called seedless grapes are of the latter type (19), whereas the navel orange is illustrative of the former situation, where not even pollination is necessary (25).

Most seedless or parthenocarpic fruits, as they are technically called, are probably produced as a result of pollination without fertilization. It was as a result of the production of seedless fruits in infertile crosses in *Oenothera* that the writer became interested in the subject. It was reasoned that if the mere pollination stimulated the ovary to grow into a fruit there must be transferred a stimulus either from the pollen or the pollen tube to the ovary, and accordingly, several experiments were set up to test the idea. Laibach (15) and Thimann (23) had found that pollen contained growth hormones or auxin. If pollination without fertilization can cause the ovary to develop into a fruit, and pollen contains auxin, why not supply synthetic hormone directly to the pistil? That was done, and in 1936 (5) the writer succeeded in producing seedless

fruits of the tomato and many other plants by applying synthetic growth hormones to the pistil. The general procedure was to mix the hormone or growth-promoting substance with lanolin, which was applied to the cut surface of the style of unopened flower buds after the anthers had been removed. To insure diffusion of the chemical into the ovary the style was shortened, and when the style was not too long the stigma was cut to allow easy penetration. The growth-promoting chemicals used in this early work were phenylacetic, indoleacetic, indolepropionic, and indolebutyric acids. Concentrations of chemicals were quite high, sometimes as high as 2 per cent in lanolin, but 0.25 per cent concentrations were also used.

In 1934 Yasuda (28) had produced a few near-normal sized cucumbers by injecting into the young ovaries of the cucumber flowers water extracts of cucumber pollen. As far as is known this was the first success in producing full-sized fruits without seeds by chemical treatment. Other investigators (10) had previously attempted to initiate fruit development by treating the pistil with pollen extracts, but the growth produced was limited.

In 1937 Gardner and Marth (3) produced parthenocarpic fruits in *Ilex opaca* and the strawberry with hormone treatment. In the same year Hagemann (11) produced seedless fruits in the gladiolus. Since that time numerous investigators have grown parthenocarpic fruits as a result of treating the pistils with growth hormones, but there have been many failures too. No success has been obtained with such plants as apples, pears, cherries, and peaches.

A practical use of this method, which was not anticipated in 1936, has also been made. Among the investigators most responsible for this may be mentioned Howlett (12), Strong (22), Roberts and Struckmeyer (20) and Murneek and Wittwer (18). During the dark months of December, January, and February, the setting of tomatoes grown in greenhouses in the northern states is very much reduced due to the poor development of pollen. By spraying the blossom buds with any one of a number of growth-promoting substances the setting has been increased as much as one hundred per cent, and the fruits are of normal or even larger size. Some investigators have added a spreader to the solution, which enables the chemical to remain spread over the surface of the blossom buds after the water has evaporated, but others have dissolved the chemical in alcohol and made dilutions of this concentrated

Figure 1. John Baer tomato fruits. The two central fruits were produced by pollination and the two end ones by treating the cut style of an unopened flower bud with 2 per cent indolebutyric acid in lanolin.

Figure 2. A cross section of a parthenocarpic tomato fruit produced by treating cut style of an unopened flower bud with indolebutyric acid. The locules are well developed, but the ovules have not developed into seeds. The gelatinous material found in normal seeded fruits fills the locules of this parthenocarpic fruit.
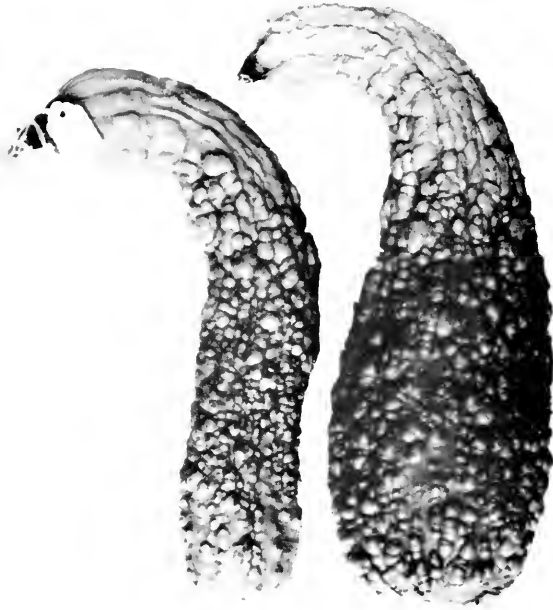
Figure 3. Fruits of crook-neck summer squash. *Left:* Parthenocarpic fruit produced by the application of indolebutyric acid to the style of flower bud. *Right:* A normal seeded fruit produced by pollination.
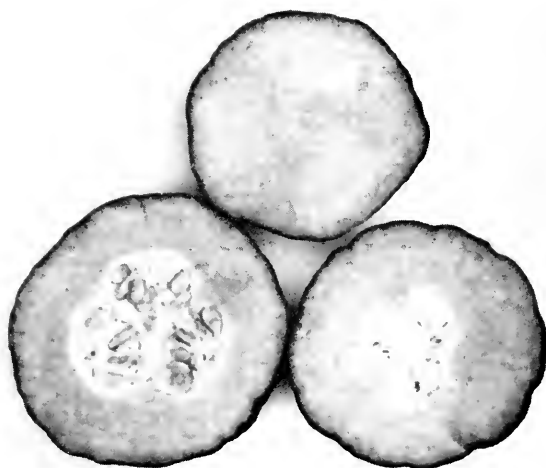
Figure 4. Buttercup Squash. The fruit at the lower left was produced by pollination and has an abundance of seeds. The other two are seedless and were produced by treating the pistil with 2 per cent naphthaleneacetic acid in lanolin.
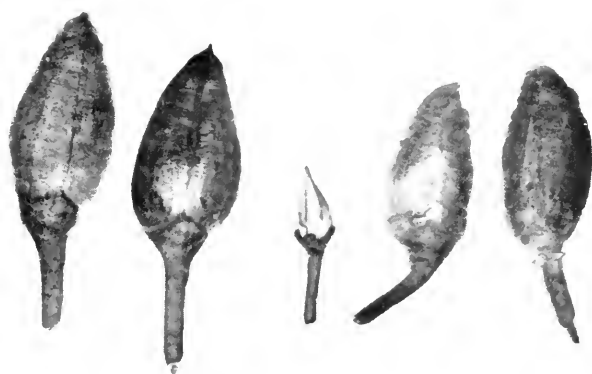
Figure 5. Fruits of Maryland Mammoth Tobacco. *Left:* Two normal fruits produced by pollination. *Center:* The ovary of a freshly opened flower. *Right:* Two parthenocarpic fruits produced by injecting a solution of 0.2 per cent potassium indoleacetate into the ovary through the pedicel.

Figure 6. Crook-neck Summer Squash. At the extreme left is a normal fruit. All the others were produced by cutting off the apical end of the ovary in the flower bud stage and smearing the cut surface with 5 per cent indolebutyric acid in lanolin. The larger ones at bottom and right had more of the ovary left and some ovules.

solution. Mitchell *et al.* (17) have used carbowax in a concentration of 0.5 per cent with good results. Concentrations of growth substances have ranged from a few parts per million to 300 ppm., but part of this difference in concentration has been due to the type of chemical used. Wittwer (26) has found that 25 to 30 ppm. of *p*-chlorphenoxyacetic acid gave satisfactory results. Not only has it been found that hormone spray increases the setting of tomatoes in the greenhouse during the winter, but it has also been found that if the first two or three clusters of flower buds on plants grown outdoors in early spring are sprayed the setting will be much increased. The cold of the early spring is extremely unfavorable for tomato setting, perhaps because of poor pollen development, but the pistils are perfectly capable of developing into fruits if stimulated by growth-promoting substances. The chemicals used to increase fruit-setting in the tomato are numerous, but the ones most commonly used are indolebutyric, naphthaleneacetic, and naphthoxyacetic acids as well as several derivatives of phenylacetic acid as *p*-chlorphenoxyacetic, 2,4-dichlorphenoxyacetic, and 2,4,5-trichlorphenoxyacetic acids. In passing it should be stated that in spraying tomato buds or flowers either in the greenhouse or in the field the resulting fruits are not always parthenocarpic since sometimes pollination may have taken place before the spraying, but this pollination may be so light that without the additional hormone the pistil would not develop into a fruit.

Recently two papers appeared adding another economic plant that profits by hormone spray. Blondeau and Crane (1) and Stewart and Condit (21) have reported that Calimyrna figs, which are usually produced only as a result of pollination by the fig wasp, produced parthenocarpic fruits of normal size and color when sprayed with 2,4-dichlorphenoxyacetic, 2,4,5-trichlorphenoxyacetic, or indolebutyric acids. These authors state that if the spray method proves to be adaptable to commercial groves it will be of inestimable value to the California fruitgrowers, as there will be no further need of growing the pollen-producing caprifig trees.

Figures 1 to 5 illustrate typical parthenocarpic fruits produced by the writer. Figure 1 of the John Baer tomato shows that externally there is no difference between the seeded and seedless fruits; and in Figure 2 it is seen that internally the parthenocarpic fruit is like the normal fruit in that it is fleshy, possesses prominent locules, but the ovules have

enlarged only slightly. In the crook-neck summer squash (Fig. 3) the ovary developed into a fruit of normal length without fertilization, but when no seeds were produced the locular region did not grow as extensively as when seeds were produced. The result was that the parthenocarpic fruits were quite long and thin and showed none of the bulging of the seeded fruit. Figure 4, of buttercup squash, brings out the fact that sometimes the resulting fruit may lack entirely the locules and be composed only of solid flesh. This has also been found in the tomato. The tomatoes lacking locules are, as a rule, considerably smaller than the normal seeded fruits. The writer has also observed this in naturally occurring parthenocarpic avocados, where the stone is lacking.

Parthenocarpy in nonfleshy fruits is illustrated by the Maryland Mammoth tobacco shown in Figure 5. In this plant the pedicel of the flower is quite stout, and it was possible to inject a solution into the ovary through the thickened pedicel. Ovaries injected with a solution of 0.2 per cent potassium indoleacetate grew more during the first five days after injection than the ovaries from pollinated flowers, and reached nearly the same final size as the seeded fruits.

Janes (13,14) made a comparative chemical study of parthenocarpic and seeded fruits of the tomato and pepper. He found that in the tomato total sugar and starch was greater in the parthenocarpic fruits. During early stages of development the titratable acidity was the same in both types of fruits, but during the ripening period the seeded fruits had a higher concentration and this was especially noticeable in the locular region. In the ripe fruits the percentage of dry weight was a little greater than in the seeded fruits. The mature parthenocarpic peppers had a slightly higher per cent of dry weight, soluble solids, sugars, and total nitrogen than the seeded fruits, but the difference was not great.

Gardner and Kraus (4) found in their extensive anatomical study of the development of the parthenocarpic fruits of *Ilex opaca* that with the exception of the lack of the seed there was no difference between the seeded and seedless fruits. In tobacco ovaries injected with 0.2 per cent potassium indoleacetate the ovules grew to nearly one fourth the size of ripe seeds and there was some growth in the embryo sac, but no seeds with an embryo ever formed (6). Several investigators have reported that the ovules may grow into empty seeds of considerable size (27,9).

Dollfus (2) found that ovules supply all or most of the hormone

necessary for the enlargement of the ovary into the ripe fruit. When he removed the ovules little growth took place; but if lanolin containing indoleacetic acid was placed in the locular cavity in place of the ovules nearly normal growth occurred. Gustafson (6) corroborated this finding with the crook-neck summer squash. He cut the ovary from unopened flower buds at different distances from the base. By this procedure the base was left without any ovules or with smaller or larger numbers depending upon the position of the cut. When no ovules were included in the ovary the growth was slight, and the amount of growth increased with the number of ovules left. Indolebutyric acid in lanolin smeared on the cut surface caused the portion of the ovary without any ovules to grow as much as it would have grown in a normally fertilized ovary (Fig. 6). Both of these investigators emphasized the importance of ovules and seeds in the development of fruits. Meyer (16) and also the writer (8) have shown that ovules, placentae, and seeds are much richer in growth hormones than other parts of the ovary or fruit.

As a result of these investigations the theory was developed (7) that fruit growth is initiated by the growth hormone brought into the ovary by the pollen tubes carrying the sperm nuclei into the embryo sacs. In plants like the navel orange, lemon, and grape where seedless fruits are normally produced, the growth-hormone concentration in the ovary was found to be greater than in similar varieties, which required pollination or fertilization for fruit production. Van Overbeek (24) has questioned the suggestion that growth hormones are supplied to the ovary by the pollen tubes, and he suggested the alternative that pollen tubes might carry into the ovary enzymes, or more specifically, prosthetic groups which would form enzymes, that acted on bound growth hormones to release the active form.

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The Growth Hormone Mechanism

in Fruit Development

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Tʜᴇ studies of Gustafson (5,6) and others on parthenocarpy indicate that growth hormones control the development of fruit. The mechanism whereby this control is implemented in the normal process of pollination and fertilization is incompletely known, however. Relatively few investigations of this aspect of auxin physiology have been made and their results are not in complete agreement. Undoubtedly some variation exists among plant species as is indicated by the differences in degree of natural parthenocarpy; yet, before ascribing different mechanisms to the species investigated, the differences which have been reported must be evaluated on the basis of the techniques used in the assay of auxin.

Since the normal stimulus for fruit development is fertilization subsequent to pollination, it follows that the pollen may furnish directly the growth hormones which cause the enlargement of the ovary, or it may furnish a part of a system responsible for the production of the hormone in the ovary. Laibach (8) first identified auxin in extracts of pollen of several orchids and *Hibiscus* and later (9) reported that extracts of *Cucurbita* pollen were active in the *Avena* test but those of a number of other species were not. Extracts of pollen of *Sequoia* (18), *Zea* (10,12,13), and *Helianthus* (10) have been shown to contain auxin. Laibach and Meyer (10) report relatively large amounts of auxin in both unripe anthers and ripe pollen of corn, and Wittwer (21) found that ether extracts of mature corn pollen were five times as active as extracts of immature anthers. This sequence of ontogenetic changes in the hormone content of pollen is different from that reported by Hatcher (7) for rye in which water, phosphate buffer (pH 10), and N/50 NaOH

extracts of green, unripe anthers gave much higher auxin yields than similar extracts of yellow, ripe anthers, and extracts of mature pollen grains did not contain active hormones. By grinding the mature pollen grains with glass and acidifying the mixture before extraction with chloroform, auxin is obtained from the pollen of *Nicotiana tabacum*, *Antirrhinum majus*, *Cyclamen persicum*, and *Datura suaveolens* (15). Usually more auxin is obtained if the pollen grains are germinated on 1 per cent agar containing sucrose before extraction, and if hydrolysis with 1.0 N NaOH precedes extraction of the ground pollen grains, the yields of auxin are uniformly high. The pollen of all plants probably contains auxin, but it may be present in variable amounts as free, bound, or precursor forms, and in some species several auxins may be present. The extraction procedures used are responsible for the unsuccessful attempts to obtain auxin from pollen which have been reported.

The growth hormones of the pollen are not the principal hormones involved in the enlargement of the ovary as is shown by a comparison of the amount of auxin present in the ovary after fertilization and the amount present in the pollen of a normal pollination. The assay of auxin in the ovary can be accomplished by placing the tissue on an agar block and allowing the auxin to diffuse into the agar for 2 or 3 hours. Numerous determinations of diffusible or free auxin in pistils of *Nicotiana tabacum* have shown that relatively small quantities are present at full anthesis (14). Similar results are obtained with pistils of *Antirrhinum* and corn. Following pollination in *Nicotiana tabacum* an increase in diffusible auxin is detected in the style accompanying the penetration of the pollen tubes and when fertilization occurs there is a marked increase in the amount of diffusible auxin in the ovary. The amount found in the style is 30 times the maximum amount obtained by extraction of the pollen and the amount in the ovary is 100 times as great (15). Thus the stimulus for fruit development provided by the pollen is something other than the growth hormones which it contains.

The high level of diffusible auxin content is maintained in the ovary of *Nicotiana* for at least 45 hours following fertilization although a downward movement from the ovary to the stem must occur since considerable amounts of auxin can be diffused from the pedicel alone (14). This auxin inhibits the development of the abscission layer (11,16) and thus one of the primary requirements for the development of the fruit is fulfilled.

These results indicate that following pollination and fertilization an auxin production mechanism is established in the ovary which brings about the enlargement into the fruit.

A similar sequence of changes in hormone content of the ovary occurs in *Heliopsis laetis* according to Söding (17). The amount of diffusible auxin in the flower head increases from the bud stage until the flowers begin to turn yellow then decreases until no detectable auxin is present when the flowers are fully open. After fertilization diffusible auxin again is present in the flower head but the amount decreases as the fruits mature. Söding inferred that the free, diffusible auxin of the young fruit is transformed into the immobile reserve auxin of the seed.

According to Hatcher (7) no diffusible auxin is found in the rye grain until three weeks after pollination whereupon a marked increase occurs with a maximum at five weeks, and then a steady decline follows to maturity. He concludes that the growth rate of the rye grain is not related to the amount of auxin present in the grain since very little auxin is present during the first three weeks of its development and maximum amounts are present when its growth has ceased. However, the most important auxin-growth relationship in the development of the fruit of *Nicotiana* appears to be the initiation of the enlargement associated with the presence of relatively large amounts of diffusible, free auxin as shown in Table 1. Enlargement of the pollinated pistil begins promptly when the pollen tubes reach the ovules (approximately 15 hours after pollination) at which time there is a large increase in diffusible auxin, and during the following 40 hours the ovary nearly doubles in size whereas the ovary of the unpollinated pistil enlarges very little. It should be realized that changes in the auxin content of a head of flowers or a capsule containing hundreds of ovules represent composite effects of

TABLE 1

Average length of ovaries of *Nicotiana tabacum*

HOURS AFTER ANTHESIS	POLLINATED	UNPOLLINATED
15	5.0 mm.	5.0 mm.
30	5.2 mm.	5.1 mm.
45	5.6 mm.	
60	7.5 mm.	5.1 mm.
85	11.5 mm.	5.3 mm.

fertilization and are measurable whereas similar changes in a fruit such as the caryopsis with a single ovule might not be measurable with the techniques available.

Determinations of auxin content by extraction procedures have identified bound forms and precursor forms of auxin in addition to the free auxin measured by the diffusion technique. The results of experiments employing extraction procedures are difficult to interpret because of the uncertainty as to the auxin forms involved. Some of the investigations, however, substantiate the results of the diffusion experiments and show the production of free auxin beginning in the fruit after fertilization. Laibach and Meyer (10) extracted auxin with alcohol finding small amounts in pistils of *Helianthus annuus* before fertilization and none in those of corn. After fertilization they found a sharp increase in the amount of auxin in the pistils of both species. These results have been confirmed for corn by Avery *et al.* (1) and Wittwer (21) who found that the auxin content increases for 2 or 3 weeks following fertilization and then decreases until maturity. Extracting the auxin from the rye grain with water, alkaline phosphate buffer, and N/50 NaOH, Hatcher (7) found little or no auxin until three weeks after anthesis which agreed with the determinations by the diffusion technique.

Of particular concern here are the results of the determinations of auxin in the ovary prior to fertilization. In the investigations cited above little or no auxin in the free, combined, or precursor forms was found in rye or in corn grains with the exception of some assays of kernels of the Country Gentleman variety of corn (1). In some experiments performed by the writer the grains of a hybrid corn at the silk stage yielded only 2 or 3 degrees of curvature by the diffusion technique, but extraction of the lyophilized grains with ether containing 5 per cent water for 10 hours at 23° C. gave curvatures of 30 to 40 degrees per grain. With the same extraction procedure high yields of auxin were obtained from both fertilized and unfertilized ovaries of *Nicotiana tabacum* 75 hours after anthesis, the yield from the fertilized ovaries being greater than that from the unfertilized ovaries (Table 2). It has been shown (20) that the yields of auxin by such an extraction are probably due to the conversion of tryptophan or a similar precursor to the auxin, indoleacetic acid. Preliminary experiments indicate that both fertilized and unfertilized *Nicotiana* pistils contain an enzyme system which can convert tryptophan to auxin with remarkable facility. Thus the stimulus furnished by the

TABLE 2

Auxin yields from 20 mg. ovary tissue of *Nicotiana tabacum* 75 hours after anthesis

	ML. OF AGAR	AVENA TEST CURVATURE	CURVATURE PER OVARY
Pollinated	0.4	16.6 ± 1.5	184
Unpollinated	0.4	17.8 ± 1.7	198

pollen for the production of auxin in the ovary is not part of the enzyme system concerned with the transformation of tryptophan to indoleacetic acid, although the possibility that it might be a part of a system responsible for the formation of tryptophan is not precluded.

Further information on the production of auxin in unfertilized ovary tissue of *Nicotiana* is obtained by incubation of the tissue at varying pH levels with and without an aqueous extract of pollen as shown in Table 3. Small amounts of auxin are obtained from the ovary tissue incubated at pH 5.9 but large amounts are obtained if the tissue is incubated at pH 8.0. This is in agreement with our knowledge of tryptophan converting enzymes in plant tissues, for, as Wildman *et al.* (19) have shown, the optimal pH for the enzyme system in spinach cytoplasm is pH 7.5, and below pH 6.0 or above pH 8.5 the activity of the enzyme is greatly restricted. However, if a small amount of an aqueous extract of ground pollen (containing no detectable auxin) is added to the medium, considerable auxin is produced at pH 5.9. Apparently the auxin production under acid conditions is not one of tryptophan conversion but involves

TABLE 3

Auxin yields from *Nicotiana* tissue following incubation at 37° C. for 24 hours

	Avena TEST CURVATURE
Buffer solution, pH 5.9	
20 mg. ovary tissue	5.5 ± 1.2
Extract of 10 mg. pollen	0.0
Ovary tissue + pollen extract	15.0 ± 1.3
Buffer solution, pH 8.0	
20 mg. ovary tissue	34.6 ± 1.7
Extract of 10 mg. pollen	0.0
Ovary tissue + pollen extract	40.2 ± 3.2

the transformation of other types of precursors (3,2). The existence of such precursors in the unfertilized ovary tissue of *Nicotiana tabacum* and *Antirrhinum* is indicated by the yields of auxin obtained following hydrolysis with 0.1 N HCl as shown in Table 4. Hydrolysis of *Nicotiana* tissue with 1.0 N NaOH gives equal yields of auxin, but similar hydrolysis of *Antirrhinum* tissue does not.

Hatcher (7) proposes that for rye the auxin system of the anther is different from that of the pistil, since in the former the maximum amount of auxin is found 2 to 3 weeks earlier than in the pistil and later disappears completely while the auxin of the mature pistil can be recovered by

TABLE 4

Auxin yields from 20 mg. ovary tissue following hydrolysis

Type of hydrolysis	*Avena* test curvature	Micrograms of indoleacetic acid $\times 10^{-4}$/mg.
Nicotiana		
0.1 N HCl	10.0 ± 0.2	0.4
1.0 N NaOH	12.4 ± 0.8	0.5
Antirrhinum		
0.1 N HCl	14.0 ± 0.8	0.7
1.0 N NaOH	3.9 ± 0.7	0.2

alkaline hydrolysis. He infers that auxin-a is the principal auxin of the embryo and indoleacetic acid is the principal auxin of the endosperm, the latter being the auxin concerned in the development of the pistil and anther. However, Zimmerman and Hitchcock (22) have found that negative results for indoles are obtained when active extracts of corn pollen are tested by the Winkler method, and they suggest that auxin-a may be the principal auxin of such extracts. A comparison of the yields of auxin from pollen and ovary tissue by hydrolysis with 1.0 N NaOH or 0.1 N HCl strongly suggests the existence of several types of auxin (Tables 4 and 5). The auxin of the pollen of *Nicotiana* and *Datura* is alkali-stable, acid-labile, and therefore indicated to be indoleacetic acid whereas the pollen of *Antirrhinum* contains indoleacetic acid and an acid-stable auxin or auxin-a. Similarly in the ovary tissue of *Nicotiana* and *Antirrhinum* there appears to be both acid-stable and alkali-stable auxins. These are tentative interpretations, however, for as Bonner and

Wildman (4) have pointed out, the acid-alkali destruction test is inconclusive because of the interference by proteins and inhibitors. The possibility that different auxin types occur in the pollen and ovary tissue merits further investigation.

Remaining to be investigated are many other aspects of the mechanism of auxin production in the pistil following fertilization. The greatest need exists for the re-examination of the auxin content of the pollen and pistil of the plants previously studied, this time using uniform techniques of auxin analysis to obtain conclusive evidence as to the similarity or dissimilarity of the mechanism in different species. In the pistil of

TABLE 5

Auxin yields from 30 mg. pollen following hydrolysis

TYPE OF HYDROLYSIS	*Avena* TEST CURVATURE	MICROGRAMS OF INDOLEACETIC ACID $\times 10^{-4}$/MG.
Nicotiana		
1.0 N NaOH	30.5 ± 1.8	3.6
0.1 N HCl	0.0	0.0
Antirrhinum		
1.0 N NaOH	19.7 ± 1.0	3.0
0.1 N HCl	19.2 ± 1.0	3.0
Datura		
1.0 N NaOH	27.6 ± 1.8	4.2
0.1 N HCl	0.0	0.0

Nicotiana tabacum, and perhaps other species also, following fertilization, a system for the production of diffusible, free auxin is established. The auxin production parallels the enlargement of the fruit, greatly exceeding the amount of auxin present in the pollen. The auxin in the ovary may be formed from tryptophan or a similar precursor, the pollen furnishing a part of a system responsible for the production of tryptophan in the tissues; or the auxin may be formed from other precursors, perhaps protein in nature, a substance in the pollen bringing about the release of the auxin from its inactive combination. The activator or coenzyme nature of the effective substance in pollen remains to be established.

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Growth Substances in Fruit Setting

S. H. WITTWER

POLLINATION and subsequent fertilization are prerequisites for fruit set on most plants. In their absence the ovary will usually fail to enlarge, and abscission occurs shortly. Pollination, pollen germination, pollen tube growth, gametic fusion (embryo), and triple fusion (endosperm) are, with few exceptions, all essential for seed and fruit production. Closely associated and directly coupled with some (perhaps all) of these processes are complicated hormonal mechanisms which largely control fruit setting and bring about its eventual maturation.

Certain plant reproductive structures are loci of production and accumulation of natural growth substances. This is especially true of pollen grains and the young fruit or fertilized ovary. Literature concerned with the proposition that the included growth substances in pollen may be causal in the induction of natural fruit setting has been reviewed by Skoog (41) and van Overbeek (47). The small actual amounts of auxin in the usually few pollen grains which function in fruit formation do not indicate that pollen is the source of growth substances necessary for fruit setting. Nevertheless, the facts remain that fruit setting has been induced by pollen extracts and the exact chemical nature of growth substances in pollen continues to attract considerable interest (39,59). Perhaps of greater importance is the rapid accumulation of growth substances in the ovary following fertilization. These hormonal relationships as they exist in the corn plant and their close association with the cytogenetically important processes of synapsis and syngamy (53) are illustrated in Figure 1. Similar hormonal relationships during the ontogeny of reproductive structures in corn (3,24) and in other plants (16,23,26) have been reported. It is highly probable that normal growth and development in the fruit of most plants is initiated and continued by a

series of hormonal stimulations beginning with the auxin released in the developing gametophytes and ending with its production and accumulation in the developing fruit. The effects of the release of these natural growth substances during various phases of reproduction are frequently confined not alone to stimulating fruit setting and growth but are extended to vegetative parts as well, exerting a profound effect upon
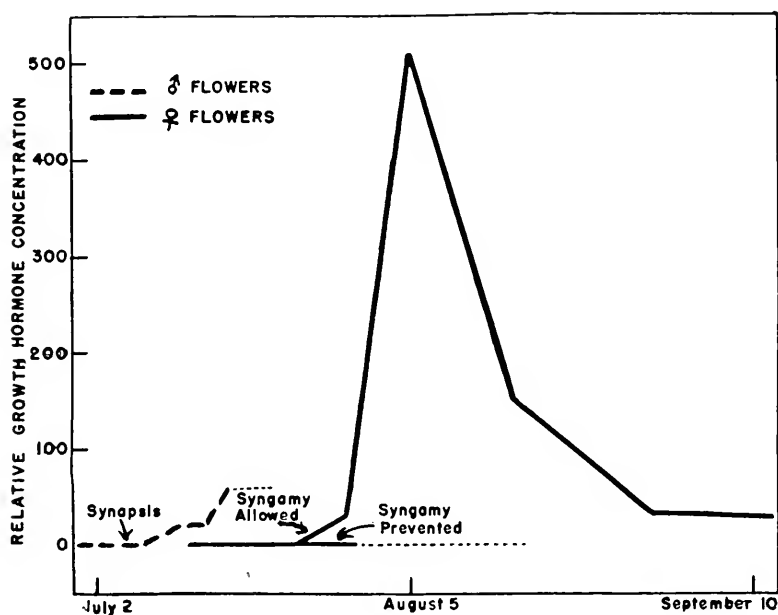
Figure 1. Changes in the growth hormone content of the reproductive organs of the corn plant during their development. There is complete absence of growth substance in the male inflorescence prior to synapsis, and in the ovule before fertilization. Subsequent to chromosome conjugation in the tassel, and the union of gametes in the ear, growth hormones appear in considerable quantities in these structures.

the growth and metabolism of the entire plant (53). Such mechanisms of auxin action involving intact higher plants are complicated, indeed, and can hardly be explained in terms used for describing auxin effects on excised tissues (2).

From the number of independent observations reported recently, there can be little doubt that the immediate causal factors in fruit setting are hormonal, providing nutrition is adequate. Considerable confusion

exists, however, pertaining to results that can be expected by the use of growth substances on many economic crops which exhibit from time to time difficulties in natural fruit setting. Most issues could be reconciled by a proper consideration of the effects of differing environments. To date, an almost total disregard of the importance of prevailing weather in determining fruit setting response to hormone chemicals has left a confused and somewhat distorted picture, making it extremely difficult to interpret many of the contradictory reports. The most profound increases in yield and fruit setting on crops reported as responding to the application of growth substances are obtained when the prevailing environment is not conducive to good fruit set.

Fruit Set in Tomatoes.—In at least one crop, the tomato, naturally produced hormones responsible for fruit set can be completely replaced by a great number of synthetic chemicals (18,45,58). These materials may be applied externally to the floral parts by several methods. Yet, even in the tomato the responses to hormone treatment, as measured by yield and fruit size increases, have not been consistent.

The significance of variety and season on fruit production in greenhouse tomatoes as they relate to the changing day-to-day pattern of solar radiation has been emphasized (54). The effects of these variables on yield and fruit size are presented in Table 1. That yields in the

TABLE 1

Effects of fruit setting treatments on yield and fruit size of greenhouse tomatoes as conditioned by variety and season

VARIETY AND SEASON	YIELD OF FRUIT IN LBS. PER PLANT			AVERAGE WEIGHT OF FRUIT IN OUNCES		
	CONTROL	FLOWERS VIBRATED	VIBRATION+ HORMONE*	CONTROL	FLOWERS VIBRATED	VIBRATION+ HORMONE
Spartan Hybrid						
Spring	9.5	12.0	14.0	3.5	4.7	4.8
Fall	2.3	3.7	5.2	4.3	4.4	4.3
Improved Bay State						
Spring	11.6	12.2	11.5	2.4	3.0	3.2
Fall	2.7	4.3	4.9	3.2	2.9	2.8

*Flower clusters sprayed with a mixture consisting of 10 ppm of *p*-chlorophenoxyacetic acid (ClPA) and 30 ppm. of β-naphthoxyacetic acid (NOA).

spring crop exceeded by almost 3 times those of the fall crop may be partially explained in the fact that the average daily solar radiation in gram calories per square centimeter received in the spring crop was 354.2 and 437.3 during the periods of fruit setting and fruit harvest, respectively, compared with 183.2 and 102.6, respectively, for the fall crop. Spartan Hybrid, an American Globe type of forcing tomato, responded favorably to supplementary fruit-setting treatments in both the spring and fall, while Improved Bay State, an English forcing tomato, showed no response in the spring but gave variations comparable to Spartan Hybrid in the fall. Differences in floral structure and quality of the pollen of the two types as affected by light intensities and photoperiod have been offered as possible explanations of the varied results obtained (5,20). In fruit size both varieties showed a similar response in the two crops.

The use of growth substances on outdoor tomatoes for improving yields and fruit set has resulted in varied and contradictory data. Both successes (27,29) and failures (31,34) have been reported. The usual controlling factor for early fruit setting in field tomatoes is night temperature. Exacting studies of Went and Cosper (49) under controlled environments, those of Smith and Cochran (42) on pollen germination and pollen tube growth, and our own (56) under field conditions have established that the optimal range of night temperature for fruit setting in tomatoes is 59° to 68°F. (15° to 20°C.). Temperatures below 55°F. will cause failure of fruit set, even on early varieties, and irrespective of the fact that the vines are making good vegetative growth and apparently flowering normally. Little viable pollen is produced and much of that appears incapable of normal germination and of producing tubes of sufficient strength to traverse the style. Limitations on tomato fruit set imposed by cold night temperatures (56,57) or extremely hot temperatures (29) can be overcome by using hormone chemicals.

Rather striking results have been obtained. Typical comparative data on early yields, total yield, and fruit size of hormone-treated versus nontreated plants obtained in the summer of 1948 in East Lansing, Michigan, are given in Table 2. The explanation for the unusual results in early yield is found in the low night temperatures prevailing during the month of June (Fig. 3), which delayed fruit set by 1 to 3 weeks on the nontreated plants.

The averaged effect of a hormone spray consisting of p-chlorophenoxy-

TABLE 2

Effect of hormone sprays on earliness, fruit size, and total yields of field grown tomatoes

Variety	Early Yield (Ounces per plant)		Total Yield (Ounces per plant)		Average Fruit Wt. in Ounces (First 5 Pickings)		Incidence of Blossom End Rot (No. Fruit per 40 Plants)	
	Treated*	Controls	Treated	Controls	Treated	Controls	Treated	Controls
Earliana x Valiant	48.8	24.4	257.1	294.6	5.4	3.8	2	9
Victor	41.7	13.0	171.7	248.0	5.2	4.6	5	8
Wisconsin 14	35.5	6.4	253.5	267.5	4.0	3.0		
Wasatch Beauty	25.9	3.0	205.9	207.0	5.6	4.6		
Bonney Best	22.9	4.8	247.9	279.1	4.9	4.1	2	3
Valiant	22.6	11.7	267.6	285.4	6.1	5.2		
Grothens Globe	21.7	5.4	208.3	224.0	6.0	5.1		
Gulf State Market	14.2	1.5	268.7	254.6	5.3	4.6	3	7
Stokesdale	13.2	4.4	254.2	243.4	5.1	4.4		
Norton's Stone	13.0		193.1	164.5	6.9	4.4	17	31
Indiana Baltimore	10.6	0.3	203.6	184.9	5.1	3.6	143	161
E. Santa Clara Canner	8.3	1.4	235.7	217.4	9.7	8.6	4	6
Rutgers	4.4	0.9	209.6	192.3	6.2	5.3	3	8
Garden State	4.2	1.0	208.2	205.8	7.2	6.5	37	43

*First 3 flower clusters sprayed with p-chlorophenoxyacetic acid (CIPA), 30 ppm.

acetic acid (ClPA), at 30 parts per million (ppm.) applied to the flower clusters of 14 varieties compared with respective controls in altering the pattern of production of field tomatoes is illustrated in Figure 2. Such alterations in harvest intensities should help to avoid seasonal overloading of markets. Peaks of production are leveled and the harvest time extended. The harvest pattern as illustrated for treated plants is more desirable not only for the grower of fresh market tomatoes but for processing as well.
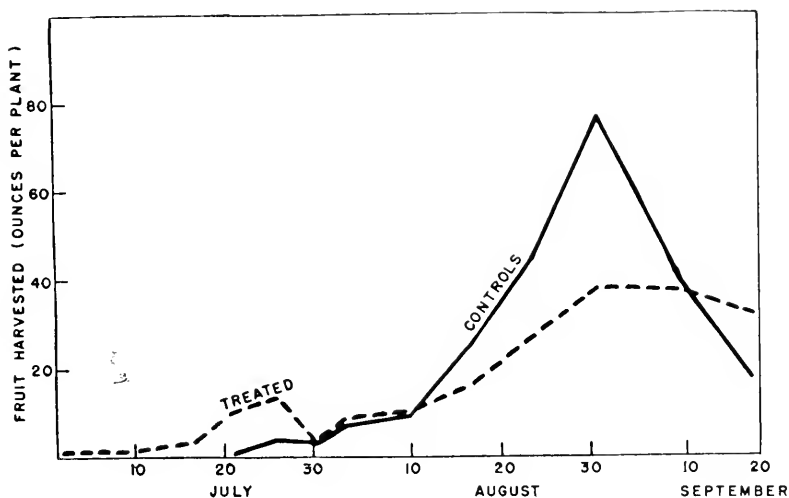
Figure 2. Comparative seasonal patterns of fruit production in hormone-treated and control tomato plants. The harvest period is extended and peaks of production are avoided when growth substances are used to overcome delayed fruit set induced by cold night temperatures.

An interesting and practical aspect of the effect of night temperatures on tomato production is the key it provides in enabling one to predict when during the harvest season the price will break on the fresh market. From the data that have been compiled, it appears that the time of the first flush of tomatoes on the fresh market is predetermined by an extended period of night temperatures favorable for fruit setting in a given area. These intervals of optimal temperature precede fruit ripening by 45 to 50 days, the usual time necessary in Michigan for fruit to ripen after it is set. In Figure 3 are given the average night temperatures prevailing during the month of June for the years of 1947, 1948, and

1949. In the three years temperatures became optimal for fruit set on the twenty-seventh, the twenty-first, and the tenth of June, respectively. Price data compiled from the combined reports of the Detroit and Benton Harbor Farmers' Markets revealed an abrupt drop of over 50 per cent in the fresh market price of tomatoes on August 13 in 1947, August 9 in 1948, and July 26 in 1949.
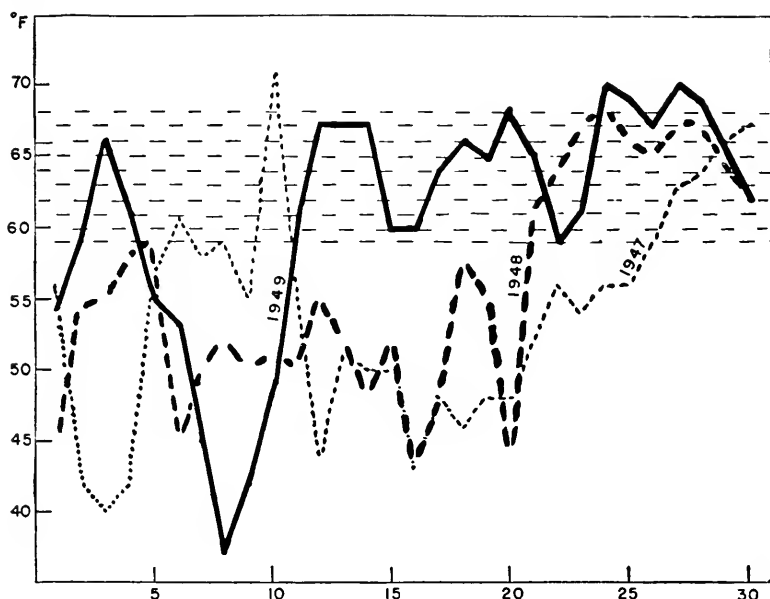
Figure 3. Averaged day to day night temperatures during the month of June in East Lansing, Michigan. Fruit setting on field tomatoes was delayed until June 10, June 21, and June 27, for the years 1949, 1948, and 1947, respectively. (Optimum range for fruit setting 59–68°F.).

Hormone sprays for greenhouse tomatoes have been used as supplementary (33) to other grower practices for improving fruit set, size, and yield. In the greenhouse temperatures are controlled so that some pollination and fertilization occurs even in very dark weather if flowers are sufficiently "vibrated." Resulting tomatoes usually contain seeds. The importance of time of application on the greenhouse crop has recently been emphasized (17,21), and present evidence indicates that pre-anthesis treatments with growth substances greatly inhibit flower bud development and are notorious for causing complete seedlessness

and the well-known defects of puffiness, green pulp in the seed locules, and premature softening. The use of whole plant sprays as compared with flower cluster sprays has at times reduced the set of fruit probably because of the inhibiting effects of growth substances upon the young flower buds (31,34). The application of growth substances to the soil (37) has been effective in setting tomato fruit, but such a technique suffers from the same disadvantages as whole plant sprays in adversely affecting the development of young flower buds.

On outdoor tomatoes when hormone chemicals are applied to the flower clusters early in the season, while night temperatures are still below 55°F., the normal reproductive processes of pollination and fertilization are totally nonfunctional. The growth substances provide a complete replacement rather than a supplement. Results as measured by early yield and size increases have been more phenomenal than any reported for greenhouse tomatoes. In the field excellent fruit set and quality is obtained, and the fruit is completely seedless. A change of weather to warmer nights, in turn, will result in normally seeded fruit, and little or no response to hormone treatment except in fruit size.

The use of whole plant sprays or dusts is desirable if growth substances are to be utilized for improving fruit set and yields on large commercial outdoor plantings. Success in their use on greenhouse crops has been reported if one avoids spraying the growing tips (33), an impractical precaution with field power equipment. Considerable work is in progress on the use of whole plant applications for canning tomatoes (57). Increases in early and total production have been obtained, but these are not always equal to those realized when the chemical is confined to the flower clusters. As a flower cluster spray, the most effective chemical has consistently been ClPA at a dilution of 30 ppm. Considerable evidence supporting this has been accumulated not only in Michigan but elsewhere (27,29). Alpha-ortho-chlorophenoxypropionic acid (ClPP) at 75 ppm. has also been relatively effective as a flower cluster spray and gives no leaf distortions or formative effects. When ClPP is used at 20 to 40 ppm. as a whole plant spray good results have been obtained, but an immediate response in fruit setting comparable to flower cluster spraying is not realized.

The incidence of blossom-end rot associated with hormone treatment of tomatoes has attracted the attention of physiologists since water relations of the plant are involved, which, in turn, may be influenced

by the auxin level (2). In Table 2 the number of fruit showing blossom-end rot is listed for both treated and control groups. It is somewhat surprising that with all varieties the incidence of the disorder was less as a result of treatment. This can hardly be ascribed to any direct effect of the applied growth substance but likely exists because nontreated plants grew more vegetatively (less correlative inhibition (30) from early fruit development), and in subsequent periods of drought their water requirement was less.

Fruit Set in Beans.—Snap beans are frequently grown in environments not favorable for fruit set. Factors which have been reported as associated with blossom drop are hot dry weather, rapid fluctuations in temperature and moisture (4,8), and, more recently, insects (14). The most striking results obtained through the use of growth substances for improving pod set have been obtained in weather with daily maximum temperatures above 90°F. and no rainfall (14,32,36,43,55). Data obtained from spray treatments of ClPA to Stringless Greenpod beans grown under such conditions of high temperatures and no rainfall are presented in Table 3. Results with growth substances on snap beans have not always been so extreme. In repeated tests under more normal temperatures (Table 3)

TABLE 3

Effects of hormone sprays on yields, pod size, and seed content of snap beans

Sprays used	Midsummer crop (Mean maximum temperatures during flowering 98 ± 3° F.)				Fall crop (Mean maximum temperatures during flowering 80 ± 7° F.)		
	Yield of pods in grams per 500 plants		Averages for total pods harvested		Total yield in grams per 500 plants	Averages for total pods harvested	
	Early	Total	Weight (Grams)	Seed Number		Weight (Grams)	Seed Number
ClPA* 1 ppm.	7,560	18,604	3.02	0.85	44,015	6.54	5.16
ClPA 2 ppm.	8,686	20,649	3.20	0.59	40,390	6.35	4.95
ClPA 5 ppm.	8,770	16,860	2.86	0.37	38,845	6.60	4.70
Controls Tap water	2,354	14,704	4.35	2.13	35,925	5.98	4.72

*p-chlorophenoxyacetic acid.

and humidity in both Missouri and Michigan, hormone sprays and dusts of ClPA and ClPP have given 10–25 per cent yield increases. Most of the increase in yield has been accounted for by larger fruit size rather than an increased set. This observation is in agreement with the recent work of Randhawa and Thompson (36). Fisher *et al.* (14), however, reported an increase due to the production of more pods rather than larger ones. Unfortunately, in snap beans precise information is lacking as to the exact temperature and humidity requirement for optimal fruit set. From a practical standpoint growth substances could be profitably used on snap beans as a type of insurance against low yields induced by unpredictable adverse weather. Consistent increases in yield of a significant magnitude and a hastening of maturity even under weather conditions favorable to pod development should further the use of growth substances in the production of snap beans.

Some of the more disappointing studies to date on the use of growth substances for improving fruit set have been with lima (7,50) and dry shell (11) beans. The hormonal stimulation of ovarian tissue adjacent to the seeds in tomatoes and snap beans resulting in increased fruit production has generally been observed to depress rather than to stimulate seed formation. When growth substances are used in a manner similar to those employed for improving fruit set in tomatoes and snap beans, they are likely to be ineffective on crops wherein yields are measured in terms of seed production, such as peas, lima beans, dry shell beans, and so on.

General Considerations.—A series of recent reports on the fig, with possible fascinating implications for other fruits, have been published (6,9,10,44). The possibility through growth substances of not only controlling fruit set but also its time of maturity as well in fruits other than the fig and, as has been reported previously, in the pineapple (48), should attract interest and stimulate research in this possible role of the plant growth hormones.

Some interest has been focused on possible alterations of nutritional values in fruits (tomatoes and snap beans) when induced to set by the use of growth substances. Variations in nutritional values have not been great nor of sufficient magnitude either to encourage or discourage the use of growth substances as fruit-setting sprays on the basis of their improvement or impairment of nutritional or market quality of the resulting produce (19,22,35,36,38,55,57). Supplementary to these in-

vestigations, Mitchell *et al.* (28) have reported a decided improvement in the retention of vitamin C and moisture in snap beans during storage when CIPA at 400 ppm. was used not as a spray designed for improving fruit set, but as a spray treatment applied four days prior to harvest. Additional studies should be made on the effects of growth substances on postharvest quality and shelf-life of such perishables as beans, peas, sweet corn, and asparagus.

The use of growth substances for improving fruit set and seed production as an aid in plant breeding has attracted an increasing interest on a great variety of crops. The work of Whitaker and Pryor (52) with melons, Schomer and Hamner (40) with berries, Emsweller and Stewart (12) with lilies, and Wester and Marth (51) with lima beans indicates that growth substances may increase both the number of successful crosses and the number of seeds per cross, and that they may also help overcome certain incompatibilities and assist in special types of vegetative propagations. Why seed production with some crops is stimulated while in other crops, and in some instances the same crop (51,55), with similar treatments it is retarded is hard to reconcile. Time and method of application are undoubtedly factors, as well as the prevention of abscission of the young fruit. Perhaps stimulatory effects of the growth substances on pollen germination and tube growth, as suggested by Eyster (13), and the data of Addicot (1), offer a partial explanation. Using identical treatments of growth substances, our results with hormone spraying of snap beans show marked decreases in seed content on some plantings and in others significant increases.

One of the most puzzling series of reports is the failure of the known growth substances in effecting fruit set on such tree fruits as the apple, pear, peach, plum, and cherry. Insofar as the author is aware, only one (46) of many who have investigated the possibilities in this field has reported positive results. Lewis (25) has summarized papers published on the stimulation of fruit development with chemicals by the statement that many-seeded fruits, such as tomatoes, cucurbits, and *Oenothera* are stimulated, whereas in few-seeded fruits, such as cherries, plums, pears, and apples, fruits are not formed. A possible exception to the rule is the snap bean pod, a few-seeded fruit, which is definitely stimulated.

Plant reproductive organs, especially the pollen and young fruit, offer interesting possibilities as source material for the isolation of new plant growth substances. Growth hormones occur in these tissues in

concentrations higher than those in any other plant tissues. Crude preparations of natural growth hormones specific for fruit setting have been prepared from the pollen and young fruit of several economic plants (15,26,45,53). Continuing efforts in the isolation of these natural growth substances should provide the key for the further elucidation of the many still baffling problems associated with the control of fruit setting.

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Growth Substances
in Pathological Growth

Experimental Induction and Inhibition
of Overgrowths in Plants

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OVERGROWTHS can be induced on plants by a variety of agents, including chemical substances, genetic factors, viruses, bacteria, fungi, nematodes, and insects. In this paper four different kinds of overgrowths induced by a chemical substance, a genetic factor, a virus, and a bacterium will be described. An account will also be given of the experimental inhibition of the growth of one of these tumors.

In 1936 and 1937 a series of papers (27,4,21) was published describing the effect on certain plant organs of indole-3-acetic acid (IAA). This substance was applied to plant organs in lanolin, the concentration of IAA varying from 1.5 to 3 per cent. The stems of both bean and tomato plants and the pods of the bean responded to this treatment with the production of overgrowths some of which had a diameter of as much as 2 cm. The overgrowths were made up in part of parenchymatous tissue and in part of the primordia of adventitious roots. Any parenchymatous tissue of the bean stem, according to Hamner and Kraus, could be rendered meristematic by this treatment.

These studies on the action of IAA on intact plants were supplemented by the investigations of Gautheret (18) on the response to this substance of plant tissue cultured *in vitro*. Gautheret showed that the response of a plant tissue to IAA depended largely on the concentration of this substance in the medium. For carrot tissue he defined its action as cambiogenic at a concentration of 0.1 mg. per liter and as rhizogenic at a concentration of 1 mg. per liter. At a concentration of between 10 and 100 mg. per liter he found that the substance ceases to be rhizogenic but induces instead a disorganized type of growth composed of hypertrophied cells.

Similar disorganized growth was observed by de Ropp (13) in fragments of sunflower stem tissue cultured on agar containing 1 mg. per liter of IAA. After a week on this medium the fragments lost their original structure and developed into shapeless, semitranslucent tissue masses. These tissue masses resembled in outward appearance the bacteria-free crown-gall tumor tissue previously isolated from sunflower by White and Braun (45). Subsequent culture of these tissues on a medium devoid of IAA showed that the change in growth pattern was not permanent. After about six months on this medium the shapeless tissue masses differentiated into roots. White and Braun (45) published similar findings in relation to the action of indoleoxaloacetic acid on plant tissues.

In the experiments quoted above the application of IAA to plant tissues did not result in a permanent change in their pattern of growth. Gautheret (18), however, has been able to isolate from carrot tissue, cultured for several years on a medium containing 0.1 mg. per liter of IAA, a strain of tissue capable of growing on a medium free of IAA without any reduction in its rate of growth. This strain of tissue was not only altered as regards its reaction to IAA, it was also changed in external appearance, having become friable and translucent instead of compact and opaque. Gautheret called the change which had taken place in this tissue "accoutomance" which can be translated as habituation. The tissue which had undergone this change he referred to as habituated tissue.

The phenomenon of habituation was also studied by Morel (30). Tissue cultures of Virginia creeper which had been grown on a medium containing 0.3 mg. per liter of napthaleneacetic acid (NAA) were transferred to a nutrient devoid of this substance. They ceased growing, but after a period of 14 months in one of the six cultures and on a limited portion of the tissue a callus developed which grew in the absence of NAA, although the rest of the culture remained unchanged. This callus on transfer continued to grow on a medium devoid of NAA.

It is difficult to explain this observation of Morel's by assuming that the NAA itself had a mutagenic action on the tissues; nor does it seem likely that anything in the nature of adaptation had occurred. The phenomenon can be more adequately accounted for by assuming that a somatic mutation occurred spontaneously in a small part of one of the tissue fragments used. This would explain why habituation occurs sporadically rather than regularly when tissues are grown in the presence

of IAA or NAA. To place these compounds in the category of carcinogenic or mutagenic agents along with such substances as the nitrogen mustards or methylcholanthrene, seems hardly justifiable at present.

The biological status of habituated tissue has been studied by Camus and Gautheret (10). Habituated tissue of scorzonera was grafted to unaltered root tissue of this plant, on which it gave rise to a voluminous neoplasm similar to that induced by crown-gall tumor tissue. These investigators favor the view that habituated tissue occupies an intermediate position between normal and crown-gall tumor tissue.

We will next consider overgrowths of genetic origin. Such overgrowths were experimentally induced by Kostoff (26) by hybridizing certain species of *Nicotiana*, notably *N. langsdorfii* with *N. glauca*. On the stems, roots, and leaves of these hybrids tumors developed either spontaneously or as a result of wounding. The overgrowths varied in appearance from fasciations such as are seen on plants affected with witches'-broom to outgrowths lacking any outwardly visible structure. Whitaker (42) attributed the formation of these overgrowths to a cytoplasmic disturbance occasioned by the introduction of the chromosome compliment of *N. langsdorfii* (used as pollen parent) into the cytoplasma of *N. glauca* (used as seed parent).

Tissue cultures of these overgrowths were made by White (44) who proved that the callus was capable of making indefinite growth on a medium containing 2 per cent sucrose, mineral salts, and yeast extract. Like habituated tissue and crown-gall tumor tissue this callus is able to grow without added auxin. Though it grew at first in the form of white, undifferentiated tissue masses, it was later shown by White (43) and Skoog (37) to be capable of differentiating roots, stems, and leaves under certain environmental conditions. White (46) also showed, by grafting fragments from cultures of this hybrid callus onto the stem of healthy *N. glauca* plants, that the hybrid tissue fragments grew under these conditions as tumors, and apparently possessed the property of propagating their tumorous nature indefinitely.

The experimental induction of overgrowths by a virus was accomplished by Black (1) in 1945 using the virus *Aureogenus magnivena*, the causal agent of wound tumor disease. This virus produces a systemic infection in a considerable number of unrelated plants into which it can be introduced either by agallian leaf hoppers or by grafting. Insects infected with the virus were found to become infectious only after an

incubation period of several days (3). Though the virus generally invaded the whole plant the tumors were local in character. Histological studies of virus tumors on sweet clover revealed that they were initiated by tangential divisions in cells of the pericycle, opposite the primary phloem (25). Abnormal cell multiplication rather than cell enlargement was found to be responsible for tumor development. The tumors were composed of a central core of xylem elements consisting of short reticulate tracheids of various widths surrounded by a meristematic zone outside of which was a layer of phloem. Tumor cells were apparently unable to differentiate into the most specialized cell type found in the xylem, the vessel, or into its counterpart in the phloem, the sieve tube. Nongranular, nonvacuolated, smooth, spherical bodies staining intensely with safranine were found in the cytoplasm of some of the tumor cells. Tumor tissue isolated from sorrel roots was successfully grown on a synthetic medium without auxin (2) and found to double its volume about every three weeks. The tissues in culture did not become organized into stems, roots or leaves. When such tissues were grafted to healthy sorrel plants the stock plants developed the systemic tumor disease, showing that the virus was still present in the tissue cultures. The heredity of the host was found to play an important part in tumor reaction. Clones of sweet-clover plants were selected which, when infected with the virus, developed minute, scarcely visible tumors; other clones were selected on which a dense mass of tumors developed. In most instances it could be shown that the overgrowth had developed as the result of a wound.

The most intensely studied of all plant overgrowths is that produced by the crown-gall organism, *Agrobacterium tumefaciens*. Erwin F. Smith who, together with Townsend, discovered the bacterial etiology of crown gall (38) emphasized the resemblance of this plant disease to animal cancer and went so far as to argue, by analogy, that some microorganism must also be associated with malignant tumors in animals. Careful study of animal cancer tissue failed to reveal any microorganisms associated causally with the cancerous condition, for which reason Smith's contention that crown gall is cancer (40,41) was rejected by most workers in the field of cancer research.

The experimental production of overgrowths on plants by means of the crown-gall organism is influenced first by the strain of the bacteria, second by the nature of the plant, and third by the manner in

which plant and microorganism are brought together. That strains of crown-gall bacteria differ in tumefacient power has been known since the time of Smith (39). Some strains exist which have lost their tumefacient power completely, others generate small galls on susceptible plants, and still others cause the formation of large rapidly growing galls. The work of Riker and others (33,28) has shown that the tumefacient power of the crown-gall organism can be eliminated by culturing it on a medium containing certain amino acids, notably glycine. Bacteria grown on a medium containing this substance lost their tumefacient power in from 10 to 20 transfers. The effect was reversible.

Crown-gall bacteria, however virulent, will produce no overgrowths unless brought into direct contact with wounded tissue. Riker (31) showed that the size of the gall produced on tomato stems was proportional to the area of waterlogged tissue resulting from the wound. He found that tissue ceased to respond with tumor production five days after it had been wounded. De Ropp (15) found that fragments of sunflower stem cultured *in vitro* also lost their capacity to respond in about this time. E. M. Hildebrandt (22), using micrurgical methods, showed that single bacteria would induce gall formation on stems of tomato plants. The size of the gall was related to the depth of the wound rather than to the number of bacteria introduced. When injected into the living surface cells of the tomato stem no galls were initiated by the bacteria, which seemed unable to survive in the intracellular environment. The work of Riker (31) and of Robinson and Walkden (36) suggests that the organisms are inter- rather than intracellular parisites.

Even when tissues are wounded they may still be unsusceptible to the tumefacient effect of crown-gall organism. In a series of experiments using slices of carrot roots inoculated with crown-gall bacteria, the writer (16) has shown that tumor formation occurs primarily along the line of the cambium. The secondary xylem which forms the core of the root is capable only of very limited tumor production. The secondary phloem outside the xylem responds rather more readily. The periderm does not respond at all. It was also shown that, in any given batch of carrot roots, grown under the same conditions and belonging to the same variety, as many as 10 per cent are generally immune to the tumefacient action of the crown-gall organism and a higher proportion are only slightly susceptible. These variations in susceptibility may be due to hereditary factors. It would probably be possible by selection to develop

highly tumorous and nontumorous strains of carrots, as has been done with mice.

The temperature at which the tissues are held after inoculation with crown-gall bacteria has an effect on tumor formation. Riker (32) found that galls developed poorly on tomato plants kept at temperatures between 28° and 30°C. and that none developed above 32°C., a finding which was later confirmed by Braun (7). The tumefacient process appears to take place within 36 to 48 hours from the time of introduction of the bacteria (6).

That plant hormones have an effect on the tumefacient process was demonstrated by Riker (34) whose observations were further extended by Braun and Laskaris (8). Tomato stems inoculated with an attenuated strain of crown-gall organisms were found to generate galls if treated at the same time with IAA in lanolin. This finding caused Braun and Laskaris to suggest that tumefaction by crown-gall bacteria takes place in two stages, the host cells being converted to tumor cells in the first stage and stimulated to continued multiplication by a growth substance in the second.

Some of the most important studies on the physiology of crown-gall tumor tissue were initiated in 1918 by C. O. Jensen (24), who showed that tumors from red beet could be transplanted to sugar beet or mangel and that, under these conditions, they continued to grow as tumors. This suggested a close analogy between the behavior of animal cancer tissue and crown-gall tissue and gave support to E. F. Smith's contention that crown gall is a plant cancer. The most significant part of Jensen's work was overlooked at the time. It consisted in the observation that crown-gall bacteria could not be isolated from tumors thus transplanted, from which he concluded that the cells of the beet tissue, under the influence of the bacteria, had developed abnormally increased proliferated power which persisted independently of continued stimulation. Smith had frequently observed that crown-gall bacteria were difficult or impossible to isolate from galls on composites, but it was not until 1943 that Braun and White (5) proved that up to 96 per cent of the secondary galls which form on infected sunflowers are devoid of crown-gall bacteria.

This discovery led to the isolation of bacteria-free crown-gall tumor tissue from secondary galls on sunflower (45). This crown-gall tissue grew indefinitely on a medium containing 2 per cent sucrose, mineral

salts, and thiamin. On this medium normal stem tissue of sunflower grew slowly, was green in color, woody in texture, and tended to differentiate roots. The tumor tissue, on the other hand, grew rapidly as a white, rather friable mass and did not differentiate organs. Bacteria-free crown-gall tumor tissue was subsequently isolated from primary galls on sunflower (36), from galls on Periwinkle that had been freed of bacteria by heat treatment (47), also from galls on marigold and Paris-daisy (23), scorzonera, Jerusalem artichoke (20,19), *Vitis vinifera*, *Opuntia*, *Carthamnus*, *Abutilon*, and *Nicotiana* (29). It seems, in fact, that these galls tend, as they grow older, to become free of the causal organism.

The isolation of bacteria-free crown-gall tumor tissue made possible a closer study of the physiology of this tissue. The respiratory behavior of crown-gall tumor tissue was compared with that of healthy tissue by White (48), who found no significant change in the qualitative respiratory picture of tumor tissue but detected a lowering of the respiratory level. The insensitivity of sunflower crown-gall tissue to indoleacetic acid, napthaleneacetic acid, and indolebutyric acid was contrasted by de Ropp (13) with the extreme sensitivity of healthy sunflower stem-tissue to these substances. Gautheret (19) working with artichoke crown-gall tissue made similar observations. Riker, Hildebrandt, and coworkers (23,35) have carried out extensive studies on the nutrient requirements of bacteria-free crown-gall tissue showing that crown-gall tumor tissue of various plants can use dextrose, levulose, and sucrose as sources of carbon for growth but have little or no ability to use organic acids or alcohols. Nitrate and urea proved to be the best sources of nitrogen of the various compounds tested. Several of the amino acids tested proved to have an inhibiting action on growth.

The influence of crown-gall tumor tissue on healthy tissue of the same plant was studied by de Ropp (12) using the technique of *in vitro* grafting. Induced tumors having a characteristic anatomical structure developed on many of the stem fragments to which tumor tissue had been successfully grafted. These induced tumors, on isolation, proved to have the physiological properties of crown-gall tumor tissue although they had apparently arisen from the cambium of the stem fragment. It was concluded that a tumefacient factor exists in crown-gall tumor tissue capable of being transferred to normal tissue across a graft union. Attempts to transmit the tumefacient principle by other means were not successful (14). Induced tumors were subsequently obtained on

healthy artichoke tissue by Camus and Gautheret (9) using the same technique. The possibility that crown gall is actually a virus disease transmitted by a bacterium was discussed by White and Braun (45), and the results of these grafting experiments lend some support to the hypothesis though they do not prove it.

It remains necessary to say a few words about the experimental inhibition of the growth of vegetable tumors. One of the most important lines of cancer research consists of a quest for a chemotherapeutic agent which will differentially inhibit the growth of cancer cells. This presupposes that there is some fundamental difference in metabolism between the cancer cell and the normal. In crown-gall tumor tissue we definitely know that such a difference exists. Crown-gall tumor tissue grows on a medium without added auxin probably because it has acquired the capacity to manufacture its own. It either has more efficient enzymatic equipment than normal tissue possesses, or it has less exacting requirements and can thus make do with a simpler set of primary nutrients.

In the course of the past year the writer has studied the effect of many substances on the growth of crown-gall tumors on carrot. These substances included so-called anti-auxins such as 2,4-dichloranisole, antibiotic substances, sulfonamides, purine and pyrimidine derivatives, and analogues of some of the B vitamins. To test these substances standard fragments of carrot cambial tissue were first inoculated with crown-gall bacteria and four days later, when the tumors were just becoming visible, were treated with 0.05 cc. of a solution containing 1 part per 1,000 of the substance to be tested. The fragments were incubated for two weeks then examined for tumors.

The anti-auxins, antibiotic substances, and sulfanamides used were found unable to inhibit tumor growth once the tumor had been initiated. Streptomycin was capable of inhibiting tumor formation but this seemed due to its action on the bacteria rather than on the tumor tissue. Complete inhibition of tumor growth was obtained with certain analogues of folic acid. These compounds had already been tested on animal cancers and found to have an inhibiting effect on the growth of tumor tissue, though their high toxicity limited their therapeutic value. The substances found most active in inhibiting the growth of crown-gall tumor tissue on carrot are issued by Lederle Laboratories under the names Aminopterin, A-methopterin, A-denopterin, A-ninopterin and A-terop-

terin. A total dosage of as little as 0.05 μg. of these substances was suffi-cient materially to reduce tumor growth (17). At low dosage levels the in-hibition could be partially reversed by a one per cent solution of folic acid. It is not yet possible to tell whether this inhibiting action is dif-ferential, that is, whether it affects the tumor cells without interfering with the growth of healthy tissue.

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In Vitro Experiments on Tissues of Pathological Origin

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THE role of growth-regulating substances in pathological growth has received wide attention. Much of this work has centered around the bacterial plant-gall disease incited by *Agrobacterium tumefaciens* (Smith and Townsend) Conn. The early literature on crown gall has been reviewed by Riker and Berge (20) and by Riker, Spoerl, and Gutsche (21). Such gall formation may result from a balance of factors among which growth-regulating substances may be important (19, 10).

Plant-tissue culture provides certain advantages over whole plants to study the fundamental aspects of normal and abnormal growth. For example, strains of callus cultures may be grown vegetatively *in vitro* for unlimited periods. The media for such cultures are easily reproducible since they initially contain only nutrients whose chemical formulae are known. This provides a fairly simple medium for closely controlled studies. Such cultures may be maintained in a relatively undifferentiated condition. In certain cases changes in the morphological character of the cultures were induced under controlled conditions. The cultures may be subjected over long or short periods to wide ranges of concentrations of supplements, including cell stimulating or inhibiting materials under otherwise constant conditions. Such cultures once established are no longer directly influenced by the association of other parts of the plant. These and other conditions seem to offer important opportunities for critical studies under closely controlled environments.

Whole plants may respond in a variety of ways to the presence of growth-regulating substances. Such responses may include formation of adventitious roots, epinasty, stimulation of cambial activity, inhibition of bud development, or delayed abscission of old leaves. A number of natural and synthetic growth-regulating compounds may induce

various ones of these effects and may either stimulate or inhibit normal or abnormal cell proliferation (see reviews in 10,28,26,23). A wide variety of natural and synthetic growth-regulating compounds have been tested on many species of whole or decapitated plants (27,34,35).

The influence of a limited number of growth-regulating substances on growth *in vitro* of cultures derived from normal tissue has been examined (18,5,6). Gautheret (5), found that indole-3-acetic acid was important for unlimited growth of tissue cultures from a number of species and was indispensable in some cases. Indolebutyric acid and α-naphthaleneacetic acid could be substituted for indole-3-acetic acid when the latter compound was essential for tissue cultures. Gautheret (6,7) reviewed the growth-regulating substance requirements of his tissue cultures as follows: 1) cultures obtained from normal carrot and endive tissue grew for limited periods *in vitro* without added indole-3-acetic acid but growth was strikingly improved by that substance; 2) cultures from normal tissues of Jerusalem artichoke, black salsify, and turnip did not grow without indole-3-acetic acid; 3) cultures of normal tissue of black salsify having once been cultivated on media with added growth-regulating substance grew in the absence of the material (The compound was required to start the culture but thereafter was no longer required even as an external source. Gautheret has referred to such cultures as habituated); 4) cultures of normal carrot tissue incubated initially on media with indole-3-acetic acid after several months developed faster in the absence of the material than did normal tissue without added growth-regulating substance; 5) Jerusalem artichoke tissue of crown-gall origin developed without added indole-3-acetic acid. Such tissues were similar to those which at first required the material but later grew in its absence. Thus, considerable information is available about the influence of added growth-regulating substances on growth and development of normal tissue. This is perhaps because generally such cultures required supplements of this kind to grow for unlimited periods, or at least they required an external source when originally isolated. This necessitated working out the proper concentration requirements.

The concentration of the growth-regulating substance is a critical item in modifying the shape and kind of cells and in determining the initiation or inhibition of root formation in the cultures of normal tissues from a number of species. For example, Gautheret (6) noted that tissue

cultures from normal artichoke failed to grow without supplementary growth-regulating substance. A concentration of 10^{-7} of indole-3-acetic acid, indolebutyric acid or α-naphthaleneacetic acid stimulated cambium formation, and a large callus was formed on the part of the tissue in contact with the medium. At a concentration of 10^{-6} the tissue continued to form cambial layers, but root formation was induced in addition. At 10^{-5} cell division and callus formation were no longer promoted, but a swelling of the cells was favored instead, and finally at 10^{-4} giant cells were produced which emphasized the action of the growth-regulating substance on the swelling of the cells. Gautheret (6) also indicated the bud-inhibiting power of the growth-regulating substances. For example, a slice of chicory root incubated on α-naphthaleneacetic acid at a concentration of 10^{-7} produced roots and a large callus, while a slice on media lacking the material produced mainly young shoots. Skoog and Tsui (24) showed similar morphological responses of normal tobacco tissue *in vitro*. They found that adenine induced bud formation in callus and stem internode tissues. Callus growth and root formation in stem segments were stimulated by α-naphthaleneacetic acid, but bud formation was inhibited. Combinations of adenine and α-naphthaleneacetic acid greatly stimulated cell proliferation and enlargement of all tissues, especially the pith, but it did not result in organ formation. A few studies have dealt with the effects of these materials on tissues of pathological origin, and have compared the responses of such tissues with normal tissues as they grew *in vitro*.

Tissues of pathological origin have been isolated from several species and studied *in vitro*. Tissues isolated from the hybrid, *Nicotiana glauca* Grah. ♀ × *N. langsdorffii* Weinm. ♂, provided the first true callus cultures capable of unlimited growth (29). This and related hybrids have been of special interest because of the type of galls (evidently of genetic origin) that are commonly produced at points of injury (16). Such galls have the general appearance of crown galls produced on a wide variety of plants by the bacterium *Agrobacterium tumefaciens*. Tissue isolated from the apical stem of the hybrid tobacco forms undifferentiated callus when incubated on solid media, but may form leaves, buds, or stems when cultured in a liquid medium. Skoog (22) found that the differentiation in liquid media was completely inhibited by the addition of 0.2 to 10 mg. per liter of indole-3-acetic acid or α-naphthaleneacetic acid. Concentrations which did not decrease the growth

rate, nevertheless prevented differentiation under conditions otherwise favorable for it. The higher of these concentrations caused a marked inhibition of growth, but at the lower concentrations the fresh weights were as large and with optimal concentrations even larger than those of the controls. Skoog also found that the cultures produced considerable quantities of auxin extractable with ether when grown either in a liquid or on a solid medium.

Bacteria-free tissue cultures of crown-gall origin from a number of species have provided for study important material of a pathological nature. Tissue cultures from primary galls on sunflower (1), periwinkle (33), Paris-daisy (12), black salsify (9), and Jerusalem artichoke (8), and from secondary galls on sunflower (31) and marigold (12) have already received considerable attention. The growth of such tissue *in vitro* removed the complicating factors that occurred when the gall tissue was studied in the intact plant. A comparison of the influence of growth-regulating substance on tissue of crown-gall origin and of normal tissue indicated some fundamental differences in response to these materials. De Ropp (2), for example, in an interesting study placed fragments of tissue of normal and crown-gall origin from sunflower and periwinkle on media containing indole-3-acetic acid, indolebutyric acid, or α-naphthaleneacetic acid at concentrations of 0.01 and 10 mg. per liter. The low and high concentrations strikingly stimulated growth of normal sunflower tissue. The low concentration resulted in decreased growth while the high concentration completely inhibited growth of the tissue of crown-gall origin. The high concentration was toxic to normal periwinkle tissues but the low concentration resulted in a slight stimulation. The periwinkle gall tissue was inhibited at the high concentration and unchanged by the low concentration. Furthermore, normal sunflower and periwinkle tissue produced abundant roots at the low concentration and the cambium proliferated at the high concentration. Exposure of the normal sunflower tissue to the high concentration of growth-regulating substance for one day was sufficient to induce root formation. These results with normal tissue were therefore similar to those described by Gautheret (5) for normal tissue of carrot and other species. The sunflower and periwinkle gall tissue showed no structural changes as a result of the growth-regulating substances.

Gautheret (8) also compared the responses of normal tissue of crown-gall origin of artichoke. Indole-3-acetic acid resulted in progressively

increased weight of the normal cultures with corresponding increases in concentrations from 10^{-8}, 10^{-7} . . . 10^{-5}. The greatest weight appeared at a concentration of 10^{-5}. Indole-3-acetic acid was not necessary for the culture of the crown-gall tissue, and the above concentrations had no effect on the wet weight of the cultures although a concentration of 10^{-4} resulted in a decreased wet weight as compared with controls lacking the supplement. Gautheret (9) similarly compared the responses of normal tissue, tissue of crown-gall origin, and of habituated tissues of black salsify to varying concentrations of indole-3-acetic acid. The tissues of crown-gall origin and the habituated tissues compared with the controls lacking indole-3-acetic acid were unaltered with respect to the increase in wet weight at concentrations from 10^{-8}, 10^{-7} . . . 10^{-5}, but were inhibited at a concentration of 10^{-4}. Both these tissues are capable of unlimited growth on media lacking growth-regulating substance. The normal tissue however was strikingly stimulated as evidenced by the progressively increased wet weight through progressively increased concentrations of indole-3-acetic acid from 10^{-8}, 10^{-7} . . . 10^{-5}. The results with the salsify normal and crown-gall tissues were therefore comparable to similar tissues of Jerusalem artichoke.

Recently Kulescha and Gautheret (15) compared the amount of growth-regulating substance elaborated by the three strains of tissue from black salsify. Ether extracts were prepared of the three tissues and *Avena* tests made in the usual manner. On a wet-weight basis normal root tissues extracted in January during the resting period contained activity corresponding to 0.5×10^{-8} of indole-3-acetic acid. Similar tissue isolated and placed on a medium-lacking growth-regulating substance had after seven days activity equal to 0.1×10^{-8} indole-3-acetic acid. Such tissue that produced buds after 10 days had an activity equivalent to 1.5×10^{-8}, but if the buds were removed the concentration decreased to from 0.2 to 0.3×10^{-8}. Tissue of crown-gall origin had much greater activity indicating a concentration equivalent to 5.3 to 5.5×10^{-8}. Similarly, the habituated salsify tissue had activity equivalent to 1.5 to 2.3×10^{-8}. The concentrations of growth-regulating substance therefore in these latter two tissues corresponded to the concentrations found earlier to be optimum for callus formation in tissues from a variety of species (5) and suggested why such tissues required no supplementary growth-regulating substance. Tissues of crown-gall origin and habituated tissues had similarities and differences as compared

to normal tissue with respect to gall production when each, respectively, was grafted to healthy plants.

Tissues of crown-gall origin from a number of species, after cultivation *in vitro*, when grafted back to healthy plants commonly continue to grow and produce a gall at the graft cite. This was first described by White and Braun (31) for sunflower tissue isolated from secondary crown galls. Such tissue was also grafted successfully to Jerusalem artichoke plants. The gall tissue itself actually increased in size, the host plant providing a suitable medium and support. This same phenomenon of grafting was successfully demonstrated for the hybrid tobacco tissue (32), for periwinkle (33), and black salsify (9). De Ropp (3,4) carried this study further with some interesting results on the interaction of normal and gall tissue in *in vitro* grafts. This approach to the role of growth-regulating substances in normal and pathological growth offers some splendid opportunities and deserves further attention.

The above information provides a general background to the field of plant tissue cultures with special attention directed toward the influence of growth-regulating substances on such cultures. Most of the work has been concerned with cultures derived from normal tissue. A few comparisons were made of the influence of these materials on tissue of normal and pathological origin. Next to be summarized are some phases of the problem studied at Wisconsin. These were concerned with the influences of some representative natural and synthetic growth-regulating substances on tissue cultures from hybrid tobacco and from secondary crown gall on sunflower. The stimulating or inhibiting effects on growth of different compounds, their critical concentrations and their action on different tissues were investigated. These items were of special interest since the stimulating and inhibiting effects on tissue cultures of sunflower and tobacco of a variety of plant tissue extracts could not be entirely explained on the basis of improved salt balances in the basal media (13,14).

Influence of concentration of growth-regulating substance on weight of cultures.—The general procedures for culturing plant tissue *in vitro* were observed. The hybrid tobacco tissue was supplied by P. R. White in December, 1941. The secondary sunflower crown-gall tissue was isolated in December, 1941, and was free of the inciting bacteria. The basal media were modifications of White's (30) and were described by Hildebrandt, Riker, and Duggar (14).

The growth-regulating substances tested included cysteine hydrochloride, indole-3-acetic acid, indolebutyric acid, p-chlorophenoxyacetic acid, α-naphthaleneacetic acid, α-naphthaleneacetamide, β-naphthoxyacetic acid, 2,4-dichlorophenoxyacetic acid, sodium 2,4-dichlorophenoxyacetate, and 2,4-dichlorophenoxybutyric acid. Each of the compounds, respectively, was added to the basal media in concentrations from 1×10^{-1}, 1×10^{-3} ... 1×10^{-13} grams per liter except indole-3-acetic acid which was not tested at 1×10^{-1} grams per liter. Controls without added growth-regulating substance were used.

Four tissue pieces were incubated in each 125 ml. Erlenmeyer flask. Each concentration was "replicated" six or more times so that twenty-four or more tissue pieces were cultured on each concentration in each trial. In addition certain compounds were tested in trials made at different times of the year. The influence of the growth-regulating substance on growth was measured after six weeks incubation by removing each piece from the flask and by weighing it.

The average wet weights of cultures incubated on the varying concentrations of some growth-regulating substances are indicated in Figure 1. Only a few curves for some representative growth-regulating substances are presented to conserve space. The weights of the cultures on each of the materials tested were presented elsewhere (11). Statistical analyses of variance showed these differences between concentrations with any one compound to be highly significant.

With sunflower tissue the differences in weights varied with the concentration and with the types of growth-regulating substance. Inhibition of sunflower tissue occurred at extremely high dilutions of 2,4-dichlorophenoxyacetic acid, sodium 2,4-dichlorophenoxyacetate, 2,4-dichlorophenoxybutyric acid, α-naphthaleneacetic acid and of p-dichlorophenoxyacetic acid. An increase in wet weight resulted from high dilutions of α-naphthaleneacetic acid and indole-3-acetic acid. Strong to complete inhibition appeared with all supplements as their strength was increased. Some comparisons of the inhibiting concentrations are presented in Table 1.

With tobacco tissue none of the compounds tested resulted in any large increases in wet weights. All resulted in decreased wet weights at the higher concentrations. The comparative inhibiting concentrations are indicated in Table 1.

The dry weights of sunflower tissue cultures incubated on media
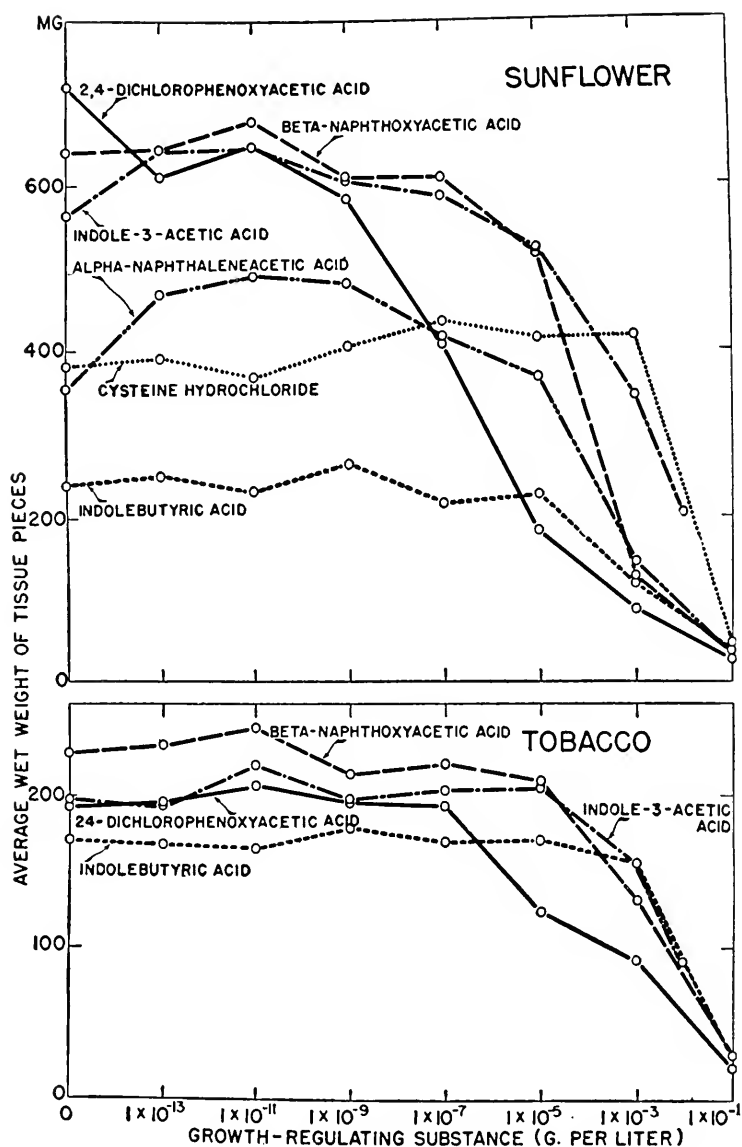
Figure 1. Average wet weights in milligrams of sunflower and tobacco tissue cultures incubated in the dark for six weeks on basal, synthetic media supplemented with different concentrations, respectively, of growth-regulating substances as indicated. Zero concentration indicates the control cultures with no added growth-regulating substance. Each point represents the average wet weight of 36 to 108 tissue pieces from one to three experiments.

with varying concentrations, respectively, of *p*-chlorophenoxyacetic acid and α-naphthaleneacetic acid expressed as percentage of wet weights varied from 4.6 to 6.8. Similar experiments with α-naphthaleneacetic acid and indole-3-acetic acid, respectively, and tobacco tissue provided dry weights that ranged from 5.3 to 9.9 per cent of the wet weights. When the average wet and dry weights were plotted on a logarithmic scale the resulting curves had similar trends. The dry weight expressed as the per cent wet weight decreased as the average wet weight increased. (The lowest per cent dry weights were obtained with concentrations

TABLE 1

Comparative Inhibiting Concentrations of Some Growth-Regulating Substances on Sunflower and Tobacco Tissue Growing *in vitro*

| | INHIBITING DILUTION | |
GROWTH-REGULATING SUBSTANCE	SUNFLOWER TISSUE	TOBACCO TISSUE
	gm./l.	gm./l.
2,4-dichlorophenoxyacetic acid	1×10^{-13}	1×10^{-5}
2,4-dichlorophenoxybutyric acid	1×10^{-7}	1×10^{-3}
sodium 2,4-dichlorophenoxyacetate	1×10^{-5}	1×10^{-5}
p-chlorophenoxyacetic acid	1×10^{-5}	1×10^{-3}
indole-3-acetic acid	1×10^{-5}	1×10^{-3}
β-naphthoxyacetic acid	1×10^{-5}	1×10^{-3}
α-naphthaleneacetic acid	1×10^{-5}	1×10^{-3}
indolebutyric acid	1×10^{-3}	1×10^{-1}
α-naphthaleneacetamide	1×10^{-3}	1×10^{-3}
cysteine hydrochloride	1×10^{-2}	1×10^{-1}

providing the greatest wet weight). This suggested that stimulation of the growth-regulating substances was associated with a swelling of the tissues.

The inhibiting effect of the growth-regulating substances was more striking with sunflower tissue than with tobacco tissue. This was perhaps because the sunflower grew more rapidly. Sunflower tissue on control media increased in wet weight during six weeks to an average of 450 mg. per tissue piece as compared to only 200 mg. for tobacco tissues. The sunflower tissue also had a greater water content.

The sensitivity of these tissue cultures to extremely low concentrations of growth-regulating substances suggested some possibilities for assay of those compounds that have been difficult or impossible to assay by

other methods. For example, 2,4-dichlorophenoxyacetic acid and its sodium salt induced negative curvature on stems and leaves of sensitive plants at 1.5×10^{-2} grams per liter and modified organs at 3×10^{-3} grams per liter (34), while with sunflower tissue cultures 2,4-dichlorophenoxyacetic acid inhibited growth progressively from the lowest concentration of 1×10^{-13} grams per liter to the highest concentration. The sensitivity of this tissue to the other compounds was also greater than that observed with whole plants. The sensitivity of the cultures to some of the compounds compares with that of the *Avena* coleoptile. The exposure of the cultures to the materials over a six week period may account for the sensitivity to such minute amounts.

No macroscopic evidence was observed with sunflower and tobacco tissue of differentiation of leaves, stems, or roots. Under certain conditions such changes did occur in tobacco tissue in a liquid medium (29,22). The stimulating or inhibiting effects of low and high concentrations of growth-regulating substances described here were also reported by Skoog (22) for tobacco hybrid tissue, and by de Ropp (2) for sunflower gall tissue. The possibility that histological differences occurred in such tissues with different concentrations of these materials was examined next.

Histological effects of growth-regulating substances on sunflower gall tissue.—The influence has been tested of the concentrations of some representative growth-regulating substances on the structure of sunflower tissue of crown-gall origin (25). Cultures incubated on weak and strong concentrations of four compounds and on control media lacking supplements were studied. Cultures from weak concentrations of indole-3-acetic acid and α-naphthaleneacetic acid (1×10^{-11} grams per liter), indolebutyric acid (1×10^{-9} grams per liter) and *p*-chlorophenoxyacetic acid (1×10^{-7} grams per liter) were compared with cultures incubated on the strong concentration (1×10^{-3} grams per liter) and with the controls. The weak concentrations for the respective compounds were selected because they represented optimal concentrations. The strong concentrations were inhibiting (see Fig. 1 and Table 1).

The structure of the sunflower tissue was relatively simple. Such tissue consisted of hypertrophic, hyperplastic, and thick-walled scalariform cells. The control tissues contained all three kinds of cells. Very few mitotic divisions appeared perhaps because growth was at a minimum after the six-week incubation period. Cells with scalariform thickenings

were most frequently scattered among the hyperplastic regions and often were not orientated in the same direction as those of adjacent tracheal elements. This suggested a lack of organization.

Tissues grown at weak concentrations had larger hypertrophic cells than control pieces. The total number of cells per unit area was less, and there were many more scalariform vessels. To determine this relationship actual counts were made of the number of cells in fifty fields

TABLE 2

Comparison of the Number of Meristematic Cells and of Tracheal Elements of Sunflower Tissue of Crown-Gall Origin Grown on Media Containing Weak or Strong Concentrations of Growth-Regulating Substance and on Control Media Lacking Added Growth-Regulating Substance

GROWTH-REGULATING SUBSTANCE AND CELL TYPE	CELLS IN 50 FIELDS OF SECTIONS OF TISSUE CULTURED WITH INDICATED CONCENTRATIONS OF GROWTH-REGULATING SUBSTANCE*			
	STRONG	WEAK	NONE	L.S.D.†
	No.	No.	No.	
Indole-3-acetic acid				
Meristematic cells	3128	3790	4352	485
Tracheal elements	662	1063	713	286
Indolebutyric acid				
Meristematic cells	3104	4037	4505	481
Tracheal elements	517	969	604	264
α-naphthaleneacetic acid				
Meristematic cells	3091	4608	4285	548
Tracheal elements	613	1266	693	241
p-chlorophenoxyacetic acid				
Meristematic cells	2903	4136	4526	485
Tracheal elements	528	1050	811	255

*Concentrations indicated in text. †Number of cells required between treatment totals for significance at the 5 per cent level.

each about 1/4 sq. mm. in size in cultures incubated on weak or strong concentrations of the four growth-regulating substances and on the control media. The total numbers of cells in the tissue sections examined from these media are summarized in Table 2. These counts indicated that the weaker concentrations stimulated greater cell size and a greater proportion of scalariform vessels.

Strong concentrations of growth-regulating substances resulted in a striking decrease in wet weight as compared with controls (Fig. 1).

Both hypertrophic and hyperplastic cells were larger than those of tissues incubated on weak concentrations or on control media. Accordingly the number of cells per unit area on each of the four compounds was fewer with the strong concentration. Also the number of tracheal elements was fewer.

It appeared therefore that the strong concentration of growth-regulating substance tested stimulated a swelling of the cells. This swelling was greater between the strong and weak concentrations than between the weak concentration and the control. The greatest number of scalariform vessels was found on media containing the weak concentrations. The greater differences in the number occurred between the strong and weak concentrations than between the weak concentrations and the control.

These results were comparable to certain of those reported by de Ropp (2) for sunflower and periwinkle tissue of crown-gall origin. He reported that different concentrations of growth-regulating substances had no effect externally and internally except for differences in weight after a six-week culture period. The studies described here indicated however that internally the structure of sunflower tissue of crown-gall origin was considerably modified. The reasons for these differences are not clear.

The influence of growth-regulating substances on the respiration of cultures of crown-gall origin may provide some of the answers, but this approach to the problem has hardly been touched because of certain technical difficulties. Mitchell, Burris, and Riker (17) found that 0.002 M. indole-3-acetic acid inhibited respiration of sunflower gall tissue by sixty-eight per cent. This reduction in respiration with indole-3-acetic acid was comparable to that observed in stems, roots, petioles, and gall tissues from a number of sources.

Summing up then, growth-regulating substances induced striking effects on plant tissue cultures just as they did on whole plants. At least three general types of responses were observed. Tissue cultures from normal plants were especially responsive to these materials. With tissues from a number of species these materials were indispensable. If the growth-regulating substance was not added to the medium the piece of tissue originally isolated from the plant failed to form callus, and therefore cultures capable of unlimited growth were not established. Furthermore, cultures of tissue from normal plants of certain species required added growth-regulating substance in the medium to maintain indefinite growth. Concentration of added growth-regulating substance

was critical in determining whether the cultures continued to produce a callus mass or whether they differentiated roots, stems, and leaves. Weak concentrations of growth-regulating substance favored cambium development and cell division; a slightly stronger concentration was beneficial for cambial growth, but stimulated root formation in addition; while even stronger concentrations stopped cell division and favored cell enlargement or stopped growth completely.

Cultures of tissue from normal plants of certain species only required added growth-regulating substance in the medium to support growth of the original isolate or of the first few transfers *in vitro*. These cultures following the original incubation period on a medium containing supplementary growth-regulating substance grew indefinitely when transferred to a basal medium lacking added growth-regulating substance. Such cultures were described as habituated and were isolated from several plant species.

Tissue cultures of primary or secondary crown-gall origin from several species and tissues from the genetically unstable tobacco hybrid were capable of unlimited growth *in vitro* on media lacking any added growth-regulating substance. Different concentrations of supplementary growth-regulating substance induced macroscopic changes evident only as increased weight at optimal high dilutions or as inhibition or death at low dilutions. No leaves, stems, or roots were formed on cultures of crown-gall origin, but under certain conditions they were formed on the tobacco tissue. Different concentrations of supplementary growth-regulating substance influenced histologically the sunflower tissue of crown-gall origin.

There appeared therefore certain similarities and differences between tissue cultures from normal plants and those of pathological origin. Some of these similarities and differences in the requirements of the respective types of tissue cultures for growth-regulating substance were indicated. The growth-regulating-substance activity of the extracts of the three types of cultures in the *Avena* test was also reported to vary with the three types. Thus, the importance of growth-regulating substance for normal and pathological growth was suggested and has received considerable attention. However, the fundamental role of these materials in normal and pathological growth is still to be worked out. These and further studies with plant tissue *in vitro* may clarify the nature of the balances resulting in normal or pathological growth.

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The Interaction Between Causative Agents in

Diseased Growth

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THE basic problems, what starts off diseased plant growth and what keeps it going, are among the most fundamental in biology. Galls may be incited by various agencies including physical and chemical factors, virus, bacteria, fungi, nematodes, and insects. However, much of the basic research has been done with crown gall caused by *Agrobacterium tumefaciens* (Smith and Town.) Bergey *et al.*

Many of the numerous working hypotheses given to explain gall formations have been centered on one or another chemical substance. Such ideas have developed naturally from work on other diseases where injury and death were caused by some single factor, such as too much heat, a toxic spray, or a single microorganism. Many of the earlier studies on pathological growth have been reviewed elsewhere, for example, by Riker and Berge (16) and by Riker, Spoerl, and Gutsche (20). The activity of growth substances has an obvious bearing on diseased growth. The general subject has been covered in a previous paper (22) listing earlier reviews, and by various reports elsewhere in this symposium.

While a single extraneous factor may cause death, the growth of cells obviously is more complex. For normal growth many physical and chemical factors seem to operate in suitable balance with one another.

Our purpose in this discussion is to indicate how some of these factors may be thrown out of balance. Further we consider evidence bearing on the suggestion (15) that such a lack of balance may influence subsequent abnormal growth.

Histological picture.—Perhaps one of the easiest ways to visualize this problem is to consider what happens when crown-gall bacteria appear

in the intercellular spaces between cells that normally never would divide again. From histological studies, Riker (14) showed in 1923 that within two days the cell walls increased in thickness near the bacteria, the entire cells became larger, the nuclei apparently took up a position near the bacteria. New cell walls were laid down within four days in a plane perpendicular to the location of the bacteria. This time interval parallels that determined by Braun (2) with heat treatments. Further cell divisions followed rapidly and in a disorganized manner until a gall developed.

Activity of bacteria in culture.—What happened above perhaps may be clarified by examining what the bacteria have done in culture media. There the details have been followed with considerable precision. At the same time we recognize the difference between a culture tube of glass and a capillary space in the plant. Among the early changes the bacteria induced in culture, after the lag period, was the lowering of the oxidation-reduction potential of the medium (21). Hydrogen-ion concentration of the cultures was changed little, if any, except in certain cases, when, for example, complex nitrogen compounds were used also as carbon sources (24). Osmotic pressures became less (19) as sugar was used and viscosity was lowered (1).

Among the chemical products formed in culture in addition to carbon dioxide, the most abundant was a polysaccharide. This has been characterized through a series of investigations and shown to be toxic under certain conditions (7). Substances related in size have proved toxic in direct relation to their molecular size (8). In passing perhaps we should mention that toxic substances at sublethal concentrations frequently may be stimulating. Small amounts of various other substances have been isolated, including phosphatides (4), thiamin, riboflavin, biotin, pantothenic acid (13), and auxin. The last has received much special attention.

Growth substances in cultures and galls.—The production of growth substances in cultures of virulent and attenuated bacteria has been approximately the same when peptone containing tryptophane was a part of the medium (10). However, in some recent unpublished work with Hodgson, Tsui, and Skoog, it appears that in a synthetic medium the virulent culture produces more growth substances than the attenuated culture. This is noteworthy when correlated with the different symptoms induced by these cultures on decapitated tomato plants (9).

The virulent cultures induced large galls, and the attenuated cultures induced small galls covered with many little shoots. Recently Hodgson, Peterson, and Riker (unpublished) found that d-tryptophane inhibited the bacteria more than the attenuated culture. Schurr (unpublished) found by means of microbiological assays that the virulent culture produced more free tryptophane in a synthetic medium than the attenuated culture. Tryptophane can stimulate galls about attenuated bacteria. It is a precursor for other important substances.

The presence of growth substance in crown gall has been shown by a number of workers. For example, growth-substance responses in plants with galls were described by Locke *et al.* in 1938 (9). A substance in tomato was found in 1939 by the same workers (11) to behave like indole-3-acetic acid in the presence of strong acid and alkali. In related studies, Riker *et al.* (18) detected no difference in the amount of growth substances in tomato plants grown at $27°$ C., where galls developed well, and $31°$ C., where the galls did not develop.

Galls were shown (13) to contain more of tyrosinase, oxidase, peroxidase, and catalase than normal tissue. The question has appeared whether this excess of oxidizing enzymes had any connection with the lowering of oxidation-reduction potentials (21) by the bacteria and with the reduced oxygen uptake (12) induced by gall-stimulating growth substances.

Indole-3-acetic acid has been reported by some workers to be the cause of crown gall. The subject is reviewed elsewhere (12). It was pointed out that thus far the technique employed had not been adequate to justify this conclusion. However, the association of such material with embryonic growth, including galls, has been well established.

Chemical induction of galls.—Various chemicals have been employed to induce galls on different plants. Among these the growth substances are the most prominent. However, many other unrelated chemicals seem to be active (16). We (unpublished) have found that substances such as thiamin, ammonium carbonate, and 1,2,5,6-dibenzanthracene all will induce chemical galls under certain conditions. Thus the formation of galls seems to be a relatively nonspecific reaction. If this should be true, then the cause must involve rather broad biological phenomena.

The possibility of stimulating growth of galls with chemicals after inoculation with attenuated strains arose from the observation by Locke *et al.* (9) that virulent bacterial galls a few inches above inocula-

tions with attenuated bacteria induced large galls about the attenuated bacteria. Braun and Laskaris (3) and Riker (15) reported similar results with growth substances. With this as a background, Thomas and Riker (23) employed some 56 chemicals on five different kinds of plants. Each plant was puncture-inoculated with attenuated bacteria, then decapitated several inches above. Each cut stem was treated with one or another of the chemicals used. Records were kept of chemically induced galls, the sizes of the attenuated bacterial galls, and of growth-substance effects which were induced.

Of the 56 chemicals used 19 were found to stimulate, in varying degrees, the attenuated-bacterial galls. These substances represented a variety of different chemical compounds. No particular compound or type of compound was found exclusively to stimulate the attenuated-bacterial galls. The compounds most active in this respect were the growth substances α-naphthaleneacetamide, α-naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, 2,4-dichlorophenoxybutyric acid, and indolebutyric acid.

Most of the effective gall-stimulating substances also induced other growth responses, such as tissue proliferation, root stimulation, bud suppression, epinasty, and formative effects. However, stimulation of galls about attenuated bacteria was not consistently associated with the production of any particular growth response. Neither did all of the active gall-stimulating substances induce all of the growth responses. Consequently, stimulation of galls about attenuated bacteria appeared to be a distinct type of growth response that could be induced by a variety of chemical compounds. This suggests that no one of the known growth substances used was, by itself, the cause of crown gall.

Bacteria reisolated from the stimulated galls were still attenuated. This fact, and the wide variety of compounds capable of inducing gall stimulation, indicated that the effects of the chemicals used were probably on the host cells.

In addition to the known chemicals, a water extract of virulent bacterial cells, which had been dried while frozen, had a slight gall-stimulating effect. Galls produced from attenuated bacteria, which developed shoots in the absence of terminal and lateral shoots on the host plant, were also larger than normal.

Unbalance of factors for diseased growth.—While continuing to examine individual factors, one of which might act as a trigger mechanism to set

off a series of events, we may wisely consider the changes in growth that may arise if we have the factors necessary for normal growth in an unbalanced combination.

As already mentioned a considerable number of physical, chemical, and biological factors may induce gall formation. The relatively non-specific nature of this phenomenon is striking. From the evidence available, the bacteria in culture, bacteria in the tissue, and important growth substances all seem associated with lower oxidation-reduction potentials or reduced oxygen uptake.

Other critical factors besides respiration may include changes in osmotic pressure and surface tension as well as altered amounts of growth substances, vitamins, enzymes, irritating substances, and food materials. Any living cell, even a resting cell, that would fail to react when the normal balance in such factors is disturbed would seem to be unresponsive indeed.

With the development of plant-tissue-culture techniques we have improved means for determining the effects of known substances. A trigger mechanism might be touched off in various ways. Perhaps of more importance is the kind of a gun the trigger is on, what the source of energy may be, how much is present, and what inhibiting and directional factors may operate. Tissue cultures help with such determinations.

Much progress has already been made as discussed elsewhere in this symposium. The work with mineral salts (6), with sources of nitrogen (17), and with sources of carbon (5) all emphasize the importance not only of the particular substance but also of its concentration to make the proper balance in relation to other items. The encouragement or inhibition by certain amounts of particular substances has sometimes been quite conspicuous.

Let us summarize. In normal growth a number of factors, including growth substances, apparently operate in suitable balance. However, if these factors are out of balance in one way we can expect pathological growth of a certain kind. If these factors are out of balance in another way we can expect diseased growth of a different kind. Whether it is right or wrong, this point of view suggests many interesting experiments.

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Deformities Caused by Insects

T. C. ALLEN

THE hexapods gradually became adapted to a wide range of food hosts. They can thrive on inorganic as well as organic matter, but the greater proportion of them, however, feed upon plant life. In their habit of feeding, insects may disturb the physiology of the plant by causing a direct loss of tissue or its cell constituents by inoculating a transmissible toxic pathogen or by causing a pathological condition resulting from their feeding which is usually accompanied by toxic salivary secretions.

The latter group of insect feeders have been referred to as toxicogenic insects, or those insects which cause a pathological disturbance of tissue not ascribable to mere mechanical injury nor fulfilling the criteria necessary to establish the presence of some microorganism. The greatest number of toxicogenic insects belong to the orders Hemiptera and Homoptera.

Toxicogenic insects cause distinct deformative effects, and their capacity to produce such effects is often inherent in a particular species. Most striking in this respect are the gall-formers. With them the deformation of tissue is so specific that the species of insect concerned can be identified by the type of gall formed.

It is the opinion of many workers that gall formation is caused by the introduction of some insect-produced toxic substance. Darwin and writers before him frequently referred to chemical secretions injected by the "gall mother." Early thinking in regard to the possible causes of insect gall formation appeared long before our present knowledge of auxin behavior. During feeding it is possible that an insect could inject or withdraw active substances which would tend to increase or decrease the activity of plant responses to hormones.

Although no evidence exists showing that insects can produce a natural auxin, it is significant to note that in 1940 Link reported curvature responses in *Avena* tests from extracts of certain aphid, (15), and in addition, from extracts of plant tissue upon which insects had fed (27). More recently, Nysterakis (18,19) has reported that certain ones do secrete auxin which affects plant growth.

A review of investigations relating to this subject shows that there are certain similarities in growth changes in plants caused by hormones and the deformities in plants caused by insect feeding. In both cases enzyme activity appears to be involved. An interesting case suggesting a relationship between plant hormones and the effects of insect feeding is the fact that bean plants treated with hormones have failed to show the expression of certain insect damage.

Similarity in Growth Changes.—As has been previously mentioned perhaps the most significant evidence that disturbances in plants caused by insects and by hormones are similar is the change produced by the feeding of insect and mite gall-formers. It is the opinion of most workers interested in such insects that gall formation is caused by the introduction of substances into the plant tissue. In 1936 Felt (5) stated that stimulation by such substances was the fundamental principle in gall formation; and Martin (16) concluded in 1930 that since stem galls in sugar cane can be produced by injection of macerated leafhoppers, possibly auxins were involved. Brown and Gardner (2) state that the formation of galls caused by insects is a tissue response paralleling the response to growth-promoting substances of some plant tissues.

Further similarities in changes in plants caused by insects and by plant hormones may be seen in the development of adventitious buds and reduction of length and width of internodes which may follow the application of hormones (9,25). Ripley (21) has reported that some mirids cause malformation in young trees and a witches'-broom rather than a single stem is produced from the terminal leaf bud. A disease associated with the feeding of a cercopid on sugar cane has been described as reducing the length and width of plant internodes with a development of adventitious buds (10). A rosetting and shortening of internodes of alfalfa and clover by the feeding of spittle insects has recently been recorded (6). Smolak (24) describes a witches'-broom of lilac caused by *Eriophyes lowi* (Nel). Psyllid yellows, which is associated with the feeding of *Paratrioza cockerelli* (Sulc), possesses symptoms that include rosetting

of leaves in witches'-broom fashion at the internode. Many abnormal tissue responses have been pointed out (8,12,17) showing that the feeding of certain *Empoasca* species will cause stunting, rosetting, and proliferation of dwarfed shoots. Carter (3) observes a close analogy between the development of adventitious buds and insect galls and suggests that a possible relationship may exist between gall, hosts, and hormones. The statement by Carter that "insects feed on a specific plant species only" would suggest that auxin relations may be involved between insects and plants. It was also his opinion that if symptoms following certain insect feeding are "much more rapid than that of fungus or bacterial infection, this appears to be the principal support for the numerous suggestions in the literature that the secretions are toxic."

That the feeding of Lygus upon potato stems indicates systemic response or diffusion of a toxic principle in association with the feeding of the insect has been reported by Leach and Decker (13). Smith (23) concluded that when certain insects feed they inject toxic secretions into plants and that the saliva of Miridae is so toxic it may cause changes in the tissue much more rapidly than could be produced by a virus. This speed of action is similar to that of applied hormones, and the similarity is of considerable interest.

Similarity in Enzymic Activity where it Appears Involved.—Although the specific action of auxin may still remain to be demonstrated, there are good indications that auxin release and action are exerted in connection with enzymes.

Numerous investigators have pointed out the presence of enzymic activity in connection with insect feeding upon plants. Cosens (4) in considering the physiology of gall formation concludes that a larva secretes an enzyme which converts starch to sugar. Typical lesions caused by cotton flea hoppers were obtained by Painter (20) following injection of diastase in plant tissue, and froghopper injury to sugar cane has been shown to increase the content of oxidizing enzymes (10). Herford has reported that certain leaf hoppers secrete diastase and invertase (11); and Andrew pointed out that the saliva of some mosquitoes contains an enzyme which produces a reaction in plant juices causing precipitation of its contents (1).

Little information is available, however, on the effect of enzyme activity, insect feeding, or plant auxin on the growth and differentiation in plants. Studies by Wildman and Gordon (26) show that proteolytic

enzymes, such as tryptic extracts, trypsin, or chymotrypsin, release active auxin from isolated plant protein preparations. An effect on the plant protein resulting from insect feeding has been found by Smith (22), who has shown that proteins and amino acids, as determined by color tests, make up a major portion of the sheath material after certain insect feeding.

Suppression of Blossom and Pod Drop Caused by Lygus Species.— A similarity in the action of hormones and of insects upon plants was recently shown by the use of α-naphthaleneacetic acid to inhibit abscission of blossoms and bean pods caused by the feeding of *Lygus oblineatus* Say (7). Under field conditions the application of naphthaleneacetic acid has resulted in greater retention of number of small bean pods thereby improving the quality of the bean for market purposes.

An explanation of the above behavior may be proposed as follows. It is known that auxin inhibits leaf abscission (14). Possibly, therefore, the insect feeding stops the normal auxin supply to the base of the petiole thus permitting the abscission layer to form. The application of α-naphthaleneacetic acid would then restore the normal supply of hormone and prevent the abscission. The insect therefore may produce an auxin inactivator or interfere in some other way with the normal supply.

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Comparative Studies of Metabolism in Insect Galls and Normal Tissues

E. H. NEWCOMB

THE striking abnormalities produced by plant tissues in response to the stimuli of gall insects are familiar to all of us. On the leaves of a single hickory tree there may be, for example, as many as eight or nine different types of galls caused by as many closely related species of gall midges of the family Cecidomyiidae, while four or five kinds of midge galls can be found on the upper surface of a single leaflet. These may be tubular, conical, flask-shaped, or globular, and covered with trichomes, waxy bloom, or sugary exudate. On oaks, there is an even larger and more remarkable series of growths induced by the larvae of gall wasps or Cynipids.

The great diversity shown by galls in their tissue differentiation and arrangement, pigmentation, compartmentalization, and shape appears the more remarkable because it may be exhibited on a single plant organ by closely related species of insects ovipositing on the same surface at about the same time. Such facts have led some investigators to assume that each species of gall-forming insect must elaborate specific morphogenetic hormones to which the plant tissue responds by producing a highly specific structure. Such a viewpoint is common in current literature on galls. It must be emphasized, however, that no growth substances have been isolated and identified from the gall insects, not to mention morphogenetic compounds, and that attempts to induce gall formation by insect parts or extracts have met with little success. Recent contributions to this field have been made by several investigators whose work will be reviewed briefly.

Beck (2), studying the gall-fly *Eurosta solidaginis*, could not obtain

gall formation in *Solidago* stems with extracts of gall tissues, maggot cultures, or maggots, although he induced some gall formation by injections of trypsin, mixtures of amino acids, and protein digests. Martin (10,11), working with sugar cane, reported success in producing stem galls following inoculation of extracts of leaf hoppers and mealy-bugs producing galls on this plant normally. He also obtained galls with autoclaved extracts.

Parr (18) investigated gall formation on chestnut oak by the coccid, *Asterolecanium variolosum*, and found that 90 per cent of the injections of salivary gland extracts into young stems resulted in gall formation. The galls had the same shape as those produced by the living insects, though they were slightly smaller. Since no gall formation was obtained by injection of salivary gland extracts heated to 60°C., Parr concluded that enzymes or enzyme-like substances were the causative agents. Ptyalin and indole-3-acetic acid failed to produce galls.

The successes of Martin and Parr were achieved with insects producing relatively simple galls which belong among Küster's "kataplasmas." Küster (7) defined kataplasmas as those galls which are less highly differentiated than the normal structures on which they are borne, and are inconstant and indefinite in size and form. Prosoplasmas, on the other hand, were defined as the more specialized galls characterized by definite size and form, which in tissue differentiation are different from but not below normal. The kataplasmas include such galls as the familiar enlargements on goldenrod stems, while the prosoplasmas include most of the galls produced by gall midges and gall wasps.

Gall insects are known to secrete various enzymes, and some workers have attributed gall-forming properties to these. Küstenmacher (6), Magnus (9), and others showed that the larvae of gall insects produce diastase and invertase. Cosens (4) identified diastase in the secretions or excretions of gall insects, and believed it played an important role in gall formation. Beck (2) identified amylase, invertase, and a protease in the excrement of *Eurosta* maggots, and concluded that the proteolytic enzymes were important factors in the production of galls. In the salivary glands of the coccid, *Asterolecanium*, Parr, in 1940, found amylase, invertase, a protease, and an oxidase, but he was not able to detect peroxidase or cellulase (18). Nierenstein (15) found that tannase is produced by the larvae of the sawfly, *Pontania proxima*, which produces galls on *Salix caprea*.

Lewis and Walton (8), in a study of the formation of the cone gall of witch hazel leaves caused by an aphid, found that the stem mother injects into the young leaf cells minute drops of a substance "initiating, stimulating, and directing development and differentiation." Globules containing a crystalloid were injected by the proboscis into the cytoplasm, from which they entered the nuclei and then the nucleoli. There the crystalloids broke up into smaller bodies. For gall formation to continue normally, repeated injections of additional material were required.

Boysen Jensen (3), in reporting on experiments with larvae of a midge forming galls on beech leaves, interpreted his results to indicate the lack of a special gall-forming substance. Larvae placed on lanolin smeared on a leaf caused cell division, although when lanolin on which larvae had been held was smeared on a leaf, cell elongation resulted. It was assumed that gall formation is caused by the larva, which is guided by instinct to secrete substances similar to growth substances in definite places and in different concentrations on the leaf, thus causing the latter to produce the characteristic gall structure.

Due to the minuteness of the gall-insects and the extremely small amounts of growth substances which they could possess or inject, the isolation and chemical characterization of such substances would require enormous numbers of insects, and appears to be a formidable problem indeed. Also to be remembered is the great difficulty of rearing even a few of the frail and ephemeral gall-formers responsible for the highly organized prosoplasmas.

There is another approach to the study of galls, however, which might yield information on the nature of the insect stimulus. This is the characterization of gall metabolism and its comparison with that of the normal tissues on which the gall occurs. It is not inconceivable that knowledge of the nature and extent of change may throw light on the nature of the stimulus. Such comparative data should also aid in the interpretation of the role of certain metabolic mechanisms of normal tissue.

One such difference in metabolism results in the abnormal accumulation in galls of polyhydroxyphenols or their derivatives or condensation products. Galls may contain on a dry weight basis as much as 75 per cent tannin, and rarely contain less than 25 per cent (5). Such galls as the Aleppo and the Chinese nutgall served for centuries as a source of tannin for ink production. Both tannins and anthocyanins, as well as lignin, contain polyhydroxy aromatic nuclei. It was suggested by Wislicenus

(20) that such phenolic groupings arise by the dehydration of fructose. For example, the 2,3-enol of fructose (I) would yield pyrogallol (II):

I   II
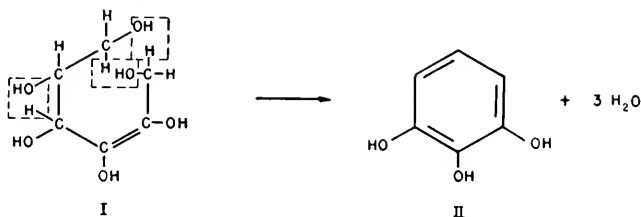
The close chemical relationship which may exist between tannins and anthocyanins is well illustrated by the formulas for the tannin, gambir catechin (I), and for cyanidin (II), the aglycone of cherry fruits, cranberries, and so on.

I   II
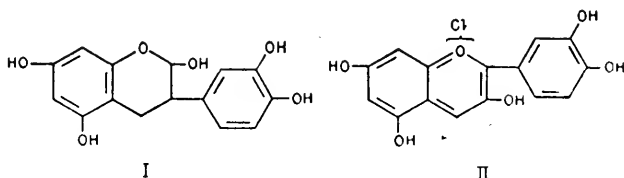
As regards the precursors of anthocyanins, many workers have substantiated the fact that there are more sugars and glycosides in leaves high in anthocyanins, and that high anthocyanin content is found especially in leaves where the transport of carbohydrate has been impeded by damaged conducting systems.

In galls such as that caused by the grape phylloxera (to be discussed later), the numbers of chloroplasts are greatly reduced so that local carbohydrate production must be small. Yet such galls are rich in starch grains in certain areas, and are high in polyphenolic compounds which presumably arise at the expense of carbohydrate. Possibly the developing galls are regions into which sugars move from the photosynthetically more active normal leaf tissue. Pertinent to this point of view is Molliard's (12) analysis of elm leaf galls produced by two species of plant lice. The galls were higher in reducing sugars than the normal leaf tissues, and were four times as rich in tannin.

Another parallel between anthocyanins and tannins lies in the correlation of high anthocyanin or tannin content with high oxidase activity. In general, the distribution of oxidase activity in flowers coincides exactly with that of the anthocyanin pigments. According to Armstrong

(1) the petals of certain flowers which have been investigated show the greatest epidermal oxidase activity in the most deeply colored varieties, less in the less deeply colored, and none at all in the white varieties.

Higher oxidase activity in galls than in unaffected tissue has been reported by several workers. Using the benzidine reaction, Parr (18) found much greater activity of oxidases in the gall tissue than in the normal oak stem tissue. The oxidases increased in activity in the gall during the growth period and then diminished. With decreasing oxidase activity in a region of the gall, the tannin content of the region increased. Molliard (12) reported increased laccase and tyrosinase activity in two elm leaf galls. Quantitative data obtained by the writer, presented in detail below, show a several fold increase of tyrosinase activity in the grape phylloxera gall during its development.

Increased oxidase activity has also been shown for bacterial galls. Nagy and Riker (13) found that the oxidase, catalase, and peroxidase activity of tomato crown gall tissue is 130, 160, and 120 per cent greater, respectively, than for the contiguous tissue, on a fresh weight basis.

Nierenstein has discovered that the striking colorations of insect galls are due to glycosides of a derivative of gallic acid, itself a constituent of tannin. It had been assumed that the gall pigments are anthocyanins, but in 1919 Nierenstein (14) reported that the pigment of a Cynipid gall on oak leaves is a glycoside of purpurogallin. The latter, which had not been reported elsewhere in nature, has the following formula:
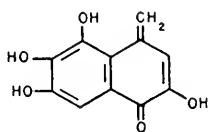
Nierenstein and Swanton (16) have shown that the pigments of a large variety of galls, caused by such diverse organisms as insects, mites, roundworms, and fungi, are all purpurogallin glycosides. All glycosides investigated yielded two glucose residues on hydrolysis. Since four biglucoside isomers are possible by attachment to one or another of the four hydroxyl groups, and two glucose residues can occur on each of two different hydroxyl groups in six different combinations to give six additional isomers, there are ten possible isomers. Eight of these were isolated from galls, crystallized, and distinguished chiefly on the basis of differences in melting points.

Nierenstein proved that Cynipid larvae secrete diastase, invertase and tannase, and postulated that the gallotannin of oak galls precipitates the first two enzymes while the tannase hydrolyzes tannin to gallic acid. Pyrogallol was presumed to arise from gallic acid by decarboxylation, and to be oxidized to purpurogallin by enzymes secreted by the larvae, the purpurogallin then being deposited in the gall as the glycoside. Although it is highly improbable that the purpurogallin, distributed throughout the gall or frequently in the superficial layers of the rind, arises through the action of enzymes secreted by the localized parasite, its widespread occurrence in and confinement to galls and its close relationship to gallotannin emphasizes the metabolic derangement in galls resulting in the elaboration of polyphenolic compounds. In confirmation of Nierenstein and Swanton's generalization as to the nature of gall pigments, the writer has isolated the purpurogallin glycoside, dryophantin, from galls produced by two species of Cynipids not examined by these authors, the acorn plum gall on black oak caused by *Amphibolips prunus* and the leaf gall on white oak produced by *Xystoteras poculum*.

The respiration of two insect galls, the large oak apple produced by *Amphibolips confluens* on leaves of red oak (*Quercus borealis* var. *maxima*), and the grape phylloxera gall produced by *Phylloxera vitifoliae* on *Vitis vulpina*, has been compared with that of the normal leaf tissue.

On a dry weight basis the rate of respiration of oak apple rind tissue ranges from about three-fourths to one-half of that of normal oak leaf tissue. The respiratory quotients of gall and normal tissues are close to unity. In both young gall and leaf disks, the respiration is reversibly inhibited up to about 50 per cent by such heavy-metal poisons as cyanide and azide, but as both galls and leaves age, the respiratory rates decline and the respiration becomes insensitive to, or rather, is even slightly stimulated by these poisons. The gall respiration becomes insensitive to the poisons earlier than the normal. The galls die in early June.

The grape phylloxera galls are initiated by young aphids on embryonic bud leaves. Within 24 hours a depression is produced with hairs at the periphery on the upper surface, and within 3 to 4 days the tissue below and around the aphid has proliferated greatly, producing a pouch projecting below the lower leaf surface and opening onto the upper surface through an orifice surrounded by trichomes. The gall reaches its maxi-

mum size in 12 to 15 days. Its morphology and development have been described by Rosen (19).

The walls and floor of the mature gall are many times thicker than the normal lamina, and the mesophyll consists of a disorganized mass of much enlarged, irregularly shaped cells. There are few stomata, and an almost complete lack of air spaces. The paucity of chloroplasts is especially noteworthy and becomes more pronounced as the galls age. Although the galls usually occur on leaf veins, their vascularization is poor due to the marked hyperplasia and the failure of small veins to develop. The expressed saps of both young and old gall and normal leaf tissues have *p*H values of from 3.0 to 3.2.

The young galls selected for study, about 2 mm. in diameter, were on young leaves 2–3 cm. long. They were compared both with normal contiguous tissue and with that of normal leaves of approximately the same age and size. In the study of mature tissues, gall activity was compared with that of contiguous normal tissue, and leaves of approximately the same size and position on the branches were used. The results in Tables 1 and 2 represent averages of several experiments.

The respiration of young and old phylloxera galls is compared with that of normal leaf tissue in Table 1. It is noteworthy that both young and old galls have a markedly lower percentage dry weight than the normal tissue. Furthermore, as the galls mature they decrease still further in percentage dry weight, although the normal tissue increases slightly. Consequently, the respiratory rates of gall tissues are higher than those of normal tissues on a dry weight basis, although lower on a fresh weight basis.

The respiratory quotients are close to unity, indicating respiration at the expense of carbohydrate. Both normal and gall cells ferment under nitrogen, the ratio of fermentative to respiratory carbon dioxide being 0.84:1 and 0.66:1 for young leaves and galls, and 0.68:1 and 0.85:1 for old leaves and galls, respectively, on a dry weight basis. While intact young galls in air show the same R.Q. as the slices, old galls, due to thickness and the impervious epidermis, show a high R.Q. indicating that oxygen is limiting the respiration, and that some fermentation is occurring (Table 1). On slicing such galls, it is found that the oxygen consumption rises and the R.Q. drops to unity.

Homogenates of gall and normal tissue were assayed for oxidase activity. Cytochrome oxidase activity could not be demonstrated in the

TABLE 1

Respiration of normal grape leaf sections, phylloxera gall slices 0.5 mm. thick, and intact galls. Temperature 25°C.

	Percentage Dry Weight		μl. O_2 uptake per gram fresh wt. per hr.		Qo_2*		R. Q.†	
	Normal	Gall	Normal	Gall	Normal	Gall	Normal	Gall
Young Sections or Slices	21.3	16.4	438	395	2.12	2.40	0.97	1.02
Mature Sections or Slices	25.3	12.9	429	268	1.46	1.75	0.96	0.99
Mature Intact Galls				160		1.24		1.40

*Qo_2 = Oxygen Uptake per Mg. Dry Wt. per Hr. †R. Q. = Respiratory Quotient.

homogenates of either young or old tissue since, when either ascorbic acid or p-phenylenediamine was used as a substrate, the addition of cytochrome c did not result in an increased oxygen consumption. On the other hand, ascorbic acid oxidase and tyrosinase activity were found in normal and gall material of all ages. The presence of ascorbic acid oxidase was shown by the vigorous oxygen consumption supported by ascorbic acid. While laccase can also oxidize ascorbic acid, the inactivity of the homogenates toward hydroquinone indicated its absence. Tyrosinase activity was shown by the oxygen consumption on a variety of poly- and monophenols known to be attacked by this enzyme. Catecholase, while not attacking monophenols, can oxidize the polyphenols employed, but its presence in any considerable amount seems unlikely because of the agreement between trends in rates of mono- and polyphenol oxidation for material of different ages (Table 2).

When gall and normal leaf tissues are compared, the trends in levels of ascorbic acid oxidase and tyrosinase activity as the tissues mature are striking (Table 2). Ascorbic acid oxidase activity is greater on a dry weight basis in both young and old galls than in leaves. As both gall and normal tissues age the activity decreases considerably.

Tyrosinase activity decreases greatly during the maturation of normal leaf tissue. But whereas tyrosinase activity in young galls is only about one-half that of young leaves, it is approximately doubled in old galls, and exceeds that of mature leaves several fold. The significance of this increase in the amount of an enzyme acting on polyphenolic compounds which takes place as the galls age is not yet clear. The fact may be recalled, however, that such compounds are capable of functioning as H-carriers with tyrosinase. Whether one or both of these oxidases participate as terminal oxidases in the respiration of these tissues is not certain. Both are copper-containing enzymes sensitive to such poisons as 8-hydroxyquinoline and allylthiourea. Inhibition of leaf respiration by these poisons is only partial (about 30 per cent for 5×10^{-2} M allylthiourea for both young and old tissue), while the gall respiration appears to be insensitive to both poisons.

The disparity in the trends which occur in the activity of the two oxidases as the gall and normal tissues mature suggests the possibility that the two enzymes are not associated in the same types of cytoplasmic particles and that there is a differential rate of multiplication of the latter. Comparison of the types and numbers of plastids and mitochondria and

TABLE 2

Substrate oxidation at 30°C. by homogenates of normal and gall tissue of grape leaves.

Enzyme	Substrate	Q_{O_2}, Young Lves.	Q_{O_2}, Young Galls	Gall Q_{O_2}, As % of Normal	Q_{O_2}, Mature Lves.	Q_{O_2}, Mature Galls	Gall Q_{O_2}, As % of Normal
Ascorbic Acid Oxidase	Ascorbic Acid	12.29	19.83	161	3.59	7.84	218
Tyrosinase	p-Cresol	3.69	1.32	36	1.25	11.00	880
	Catechol	18.10	13.15	73	6.55	32.63	498
	Dihydroxy-phenylalanine	5.64	3.29	58			
	Pyrogallol	10.27	4.88	48			
	p-Phenylene-diamine	6.83	3.46	51			

of the distribution of activity of the two oxidases between such cellular entities in both normal leaf and gall cells is now in progress. By centrifugation of homogenates of normal leaf tissue, the ascorbic acid oxidase activity has been found associated entirely with the fraction consisting of insoluble cell debris and unfragmented chloroplasts and nuclei. No ascorbic acid oxidase activity was found associated with chloroplast fragments, mitochondria, or smaller particles or soluble enzymes.

Such shifts in levels of enzyme activity may be due directly to the effects of substances injected by the insect, or to environmental modifications resultant from early changes, such as the increase in tissue thickness resulting in reduced oxygen tension within the tissue, poor vascularization, and so on. No data have been obtained for that interesting stage when the first cells affected by the aphid are dividing and differentiating to produce a recognizable gall. The earliest stages studied were of galls already possessing typical form and anatomy.

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Vitamins and Amino Acids as Growth Factors

Growth Factors in Bacterial Nutrition

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THE term "growth factor" has been used in a variety of ways. In the discussion to follow we shall use it to mean those organic factors, exclusive of the compound or compounds utilized as an energy source, which are required for growth of an organism in a given environment. This definition excludes, on purely arbitrary grounds, consideration of the inorganic nutrition of bacteria or of the variety of compounds which these microscopic plants may use to supply their requirements for energy.

Bacteria vary widely in their requirements for growth factors. Thus certain of the photosynthetic autotrophs—for example, members of the *Thiorhodaceae*—are similar to the higher plants in that they grow well in appropriate mineral media with light as their energy source and carbon dioxide as their sole source of carbon. Such organisms require no growth factors in the sense defined above. The chemosynthetic autotrophs, such as *Thiobacillus thiooxydans*, and the simpler heterotrophs, such as *Escherichia coli*, likewise require no growth factors in this sense of the term, although they oxidize either inorganic or organic materials to supply energy for synthetic purposes. A great many bacteria do, however, require organic materials other than an energy source for growth, and many organisms which do not require a specific growth factor may be greatly stimulated by it.

We may now examine the nature of the compounds which have been found to be essential growth factors for one or more species of bacteria. It will be seen from Table 1 that these represent almost all of the known water-soluble vitamins, most of the common amino acids, and a variety of miscellaneous biologically important compounds. The striking thing about this list is that with one or two exceptions to be considered later,

TABLE 1

Summary of compounds recognized as growth factors for various bacteria*

Amino acids	Vitamins	Purine and pyrimidine bases
L-alanine	p-Aminobenzoic acid	
		Adenine
D-alanine	Biotin	
	Choline	Hypoxanthine
L-arginine	Folic acid	
		Guanine
L-aspartic acid	Nicotinic acid	
	(nicotinamide)	Uracil
L-cysteine		
	Pantothenic acid	Thymine
L-glutamic acid		
	Riboflavin	**Nucleosides and Nucleotides**
Glycine		
	Thiamin	
L-histidine		Ribonucleotides
	Vitamin B$_6$ (pyridoxal	(several)
L-isoleucine	pyridoxamine,	
	pyridoxine)	Desoxyribonucleosides
L-leucine		(several, e.g.
	Vitamin B$_{12}$	thymidine)
L-lysine		
	Vitamin K	**Miscellaneous**
L-phenylalanine		Asparagine
L-proline	**Conjugated vitamins**	
		Glutamine
L-serine	Coenzyme I or II	
		Hemin
L-threonine	Pyridoxamine phosphate	
		Putrescine (spermine
L-tryptophan	Thiamin pyrophosphate	or spermidine)
L-tyrosine		
	Fatty acids	**Unidentified factors**
L-valine		
	Acetic acid	(See Table 3)
	Oleic acid (or other	
	unsaturated acid)	

*Appropriate fragments or bound forms of these growth factors may also satisfy the nutritional requirements of certain bacteria.

all of the compounds in it are of recognized importance for all forms of life so far examined. Each of them, so far as we now know, is contained in the protoplasm of every living organism, be it plant, animal, or microorganism.

Since these compounds seem to be present generally in all protoplasm, it is clear that those organisms which do not require them as nutrients must synthesize them, and that bacteria which require a preformed supply of one or more of them in the medium must, for some reason, be unable to synthesize those which they require.

This idea that the growth-factor requirements of microorganisms arose through loss of the ability to synthesize substances of importance in the metabolism of all organisms was given formal expression independently by Lwoff (19,20) and by Knight (13) about fifteen years ago, while knowledge of bacterial nutrition was still fragmentary. These authors envisaged a physiological evolution from the primitive and self-sufficient state of autotrophism toward complete parasitism, brought about by successive losses in the synthetic capacities of microorganisms. Subsequently Beadle and Tatum (2) showed that by irradiation of *Neurospora* with X-rays or ultraviolet light, mutants could be produced which showed added nutritional requirements (see also 1,3). The work of Lederberg and Tatum (17,18) and of others (16) showed that similar nutritional mutants could be produced in bacteria, and that the nutritive requirements of such mutants were for the same growth factors as had been previously shown to function in the nutrition of natural populations of bacteria. Through these developments, the theories of Lwoff and of Knight concerning the origin of nutritional requirements in microorganisms have been given a sound experimental basis. We may now view the nutritional requirements of natural populations of bacteria as having arisen by successive mutations with cumulative effects to produce organisms having requirements of varying complexity.

Since mutation is a random phenomenon it might be expected that a requirement for growth factors would be found at random among a great variety of bacterial species, and that the identity and number of the growth factors required would vary widely from one species of bacteria to another, and even from one to another strain within a single species. Such is indeed the case. As one example, the requirements of various species of the genus *Clostridium* may be cited. *Clostridium butylicum* requires only the single growth factor, biotin, for growth in

a glucose-salts medium (37). The closely related *Clostridium acetobuty-licum* requires biotin and *p*-aminobenzoic acid for growth under similar conditions (15), whereas *Clostridium perfringens* requires several different vitamins (pyridoxamine, biotin, riboflavin, and pantothenic acid), adenine and uracil, and thirteen different amino acids for growth (5). Somewhat similar variations, if not so marked, may be found in the requirements of different representatives of other groups of bacteria, for example, the lactic acid bacteria, some of which have nutritive requirements which surpass those of *Clostridium perfringens* in complexity (24,33).

It would be of little interest here to tabulate the growth-factor requirements of various bacteria which have so far been investigated. For each organism such a tabulation would show one, a few, or many of the compounds listed in Table 1 as being required. Summary articles which contain this valuable information have appeared (14,24,33). Instead, certain other aspects pertinent to this problem may be discussed.

The nature of the response to an essential growth factor is shown in Figure 1, which depicts the response of *Lactobacillus casei* to additions of riboflavin. Within limits, the amount of growth is dependent only upon the concentration of the vitamin. If, at these growth-limiting concentrations, one measures the amount of the vitamin present in the medium and the cells after growth has ceased, it is found that all of the vitamin (within experimental error) has been absorbed from the medium
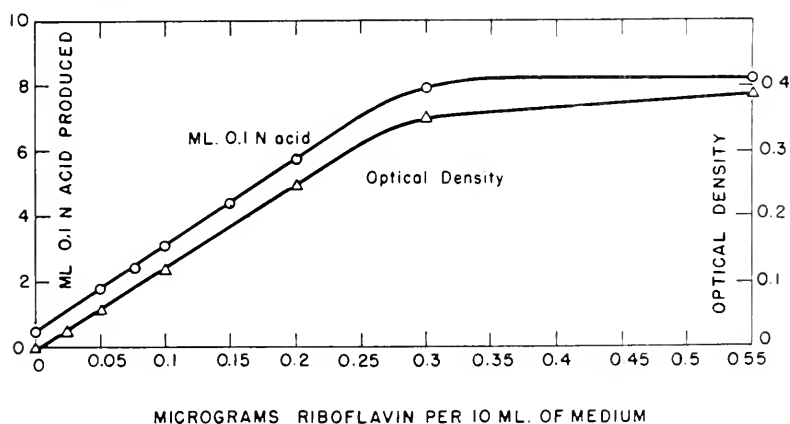

Figure 1. Relationship between the concentration of riboflavin and growth of *Lactobacillus casei*.

(35), and most of this can be recovered from the cells. This behavior typifies that of other essential growth factors. They are essential compounds for formation of protoplasm and as such are absorbed from the medium with great efficiency and incorporated into the cell. Many other closely related lactic acid bacteria synthesize the riboflavin which they require (36); *L. casei* does not and must have it preformed for growth.

In the past the inability of an organism to synthesize a growth factor has been most frequently explained by assuming that the mutation which gave rise to the nutritional requirement eliminated one of the essential

TABLE 2

The comparative requirement of *H. parainfluenzae* for purine bases in the presence and absence of guanine (9)

ADDITIONS TO BASAL MEDIUM*	TURBIDITY†
None	70
Guanine hydrochloride	94
Hypoxanthine	73
Guanine hydrochloride + hypoxanthine	74
Guanine hydrochloride + adenine sulfate	70

*100 μg. of the indicated compound to each 10 ml. of medium.
†Per cent of incident light transmitted, uninoculated medium = 100.

enzymes concerned in synthesis of the growth factor. Thus we might suppose that *L. casei* lacked one of the enzymes necessary for riboflavin synthesis. So far as is now known this is a sufficient explanation for this case. In many cases, however, this explanation does not suffice. Several examples are now known where a bacterium synthesizes a given growth factor in one medium but does not do so in another. For example (Table 2), if *Hemophilus parainfluenzae* 7901 is cultured in a defined medium which contains guanine, it must be supplied with either hypoxanthine or adenine to permit growth (9). The simplest explanation of this finding assumes that hypoxanthine (or adenine) cannot be synthesized under these conditions and consequently must be supplied to permit synthesis of nucleic acid, which is in turn necessary for growth. Yet if guanine is omitted from this medium the organism grows well in the absence of both hypoxanthine and adenine and must under these conditions synthesize its own purine bases. Failure to synthesize adenine

(or hypoxanthine) in the medium which contains guanine apparently results not from absence of the appropriate enzyme but from inhibition of some step in the synthesis by guanine. Beerstecher and Shive (4) found, similarly, that tyrosine inhibited growth of a strain of *Escherichia coli* under appropriate conditions and that this inhibition was alleviated by phenylalanine. Apparently tyrosine inhibited synthesis of phenylalanine by this organism, a synthesis which occurred without difficulty when tyrosine was omitted from the medium.

A particularly clear-cut example of a nutritional requirement which results from an inhibition rather than from lack of the enzymes necessary to carry out a synthetic process is provided by the yeast, *Saccharomyces carlsbergensis*. Under ordinary cultural conditions this yeast requires vitamin B_6 for growth, and its growth response to additions of this vitamin has been the basis for a widely used method for determining vitamin B_6. Yet if thiamin is omitted from the culture medium and the inoculum has been grown in a thiamin-low medium, the organism grows well in the complete absence of vitamin B_6 (Fig. 2), and analyses of the cells have shown that vitamin B_6 is synthesized under these conditions (25). Addition of rather small amounts of thiamin—a substance which is itself required in metabolism and is normally present in this yeast—inhibits growth, and under these conditions vitamin B_6 is required to permit growth (Fig. 2). How thiamin inhibits growth and how this inhibition is counteracted by vitamin B_6 are both unknown. The important point is, however, that a growth factor which under one set of conditions is synthesized in amounts sufficient for growth cannot be thus synthesized under another slightly different set of conditions, or is synthesized in amounts insufficient for growth. Thus the nutritional requirement for the growth factor results from an inhibition rather than from lack of the enzymes to accomplish a given synthesis.

From these examples it is evident that the failure of an organism to synthesize an essential growth factor may result either from lack of the necessary enzymes to effect the synthesis, or from inhibition of one or more synthetic processes by metabolites normally present within the cell, or added with the medium. In general terms, if b is essential for synthesis of protoplasm and is normally formed from certain precursors, a, by an enzymatic process, then the synthesis:

$$a \longrightarrow b$$

may be eliminated either by lack of one of the necessary enzymes or by

the presence of an inhibitor for the process, and in either case *b* will appear as a nutritive essential for growth, since it can no longer be formed within the cell or is formed in amounts insufficient for growth. The diagram represents only one mechanism through which an inhibitor may operate to make a previously nonessential growth factor become essential for growth. Several others can be readily visualized.
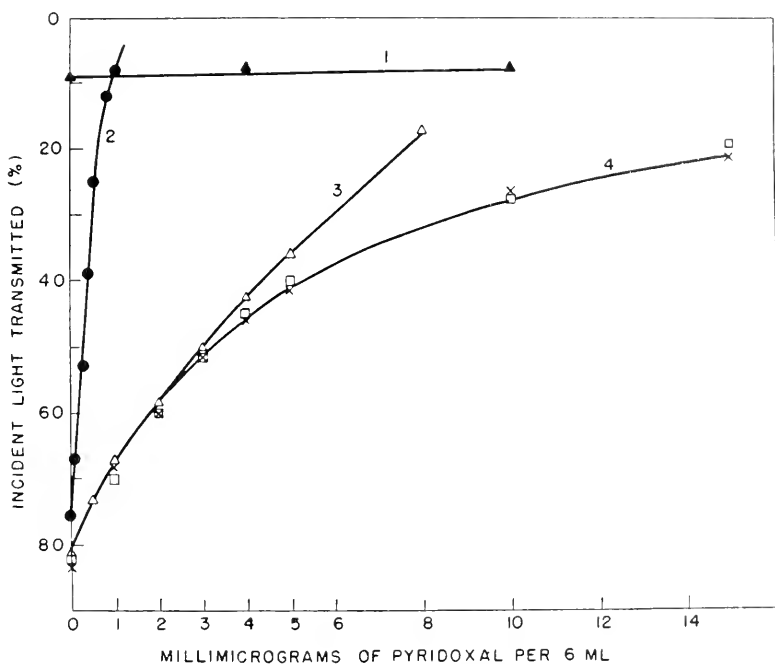
Figure 2. Inhibition of growth of *Saccharomyces carlsbergensis* 4228 by thiamin and its reversal by vitamin B_6. Curves 1, 2, 3, and 4 represent the growth response to pyridoxal in the presence of 0, 0.1, 1, or 10γ of thiamin, respectively, per 6 ml. of medium (25).

This latter explanation for the occurrence of certain nutritive requirements in bacteria has been insufficiently emphasized in the past apparently because of a disinclination to admit that normal cellular metabolites might act as inhibitors for certain metabolic reactions. Yet several such cases are now known as the few instances cited above illustrate. The concept is novel only in viewing the inhibitor as a substance normally present within the cell. It has been recognized for several

years that certain drugs act in this fashion. The sulfonamides, for example, competitively inhibit transformation of *p*-aminobenzoic acid to one or more essential catalysts in those bacteria which they inhibit. Their effects can be overcome either by addition of *p*-aminobenzoic acid, which acts competitively to overcome the effects of the drug, or by a variety of other substances (folic acid, thymine, purine bases, serine, methionine), which act noncompetitively (26,44). Each of these substances might be considered a new growth factor required by the cells under conditions where a single essential reaction has been inhibited, in this case by an inhibitor (a sulfonamide) foreign to the cell.

This concept of the origin of some nutritional requirements presents several interesting possibilities. Examples of "nutritional symbiosis" (possibly related to true symbiosis), in which each symbiont supplies by synthesis an essential nutrient for the other symbiont, are easily observed experimentally (29). Perhaps a new type of nutritional symbiosis, in which one symbiont metabolizes to its own advantage a product elaborated by the second symbiont and which would otherwise inhibit growth of the latter by preventing essential synthetic reactions, may be observed. Perhaps the toxicity of some types of organic matter for certain autotrophs may be exerted in this fashion. This point of view should also make possible a different approach to the discovery of new growth factors. For if many nutritional requirements arise naturally through the inhibition of essential synthetic reactions, then one might purposely inhibit growth of bacteria with all types of organic compounds. Wherever these toxic effects could be reversed by addition of natural materials, the possibility would present itself that synthesis of some metabolically essential compound, supplied by the natural material, was the process limiting growth in the presence of the inhibitor. Isolation of the compound or compounds effective in the reversal might then bring to light new compounds of importance in metabolism. It was in this way that *p*-aminobenzoic acid was isolated; a naturally occurring substance which overcame the toxic action of sulfanilamide was observed (45), and on isolation, proved to be *p*-aminobenzoic acid (27). More recently Shive and coworkers (30) isolated thymidine by this approach. This compound proved to be one of the naturally occurring substances which prevented the toxic action of 7-methylfolic acid for *Escherichia coli*. The approach has never been systematically exploited, however.

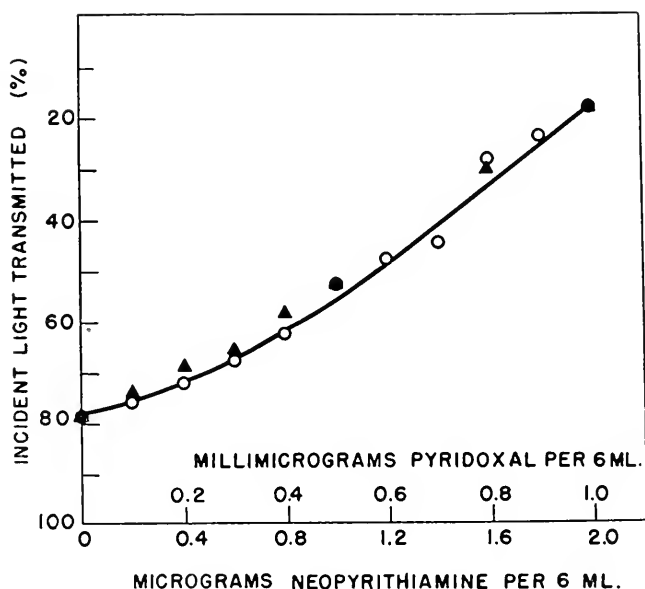We have seen that growth of *Saccharomyces carlsbergensis* 4228 is

Figure 3. Comparative growth promoting action of pyridoxal and neo-pyrithiamine for *S. carlsbergensis* 4228.

inhibited by small amounts of thiamin and that this inhibition is over-come by small amounts of vitamin B₆. In many other organisms the growth-promoting effects of thiamin can be counteracted by the anti-vitamins, pyrithiamine (46) or neopyrithiamine (43). These products are antivitamins which are so closely related in structure to thiamin (see the accompanying formulas) that although they cannot duplicate the physiological function of the latter they do interfere with these functions in a competitive manner. It was therefore of interest to determine what effect neopyrithiamine would have on an organism for which thiamin was a growth inhibitor. Here too, the product counteracted the effect of thiamin, that is, it promoted growth of *S. carlsbergensis* (Fig. 3) in the same way as did pyridoxal (25).

Thiomin

Neopyrithiamine
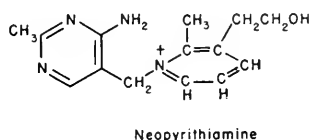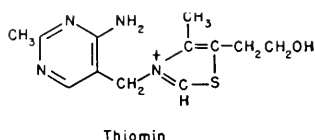
This result is of considerable interest, for it illustrates how a substance which is entirely foreign to metabolic systems may simulate the action of a true growth factor. Indeed, if these effects had been first observed in a natural medium, and if the effect of vitamin B_6 had not been known, neopyrithiamine might well have been considered as a true growth factor. It should not be considered as such despite its growth-promoting properties under these conditions, since it plays no role in the normal metabolism of this or other organisms.

Other somewhat similar examples are known. *Lactobacillus bulgaricus* for example, requires oleic acid (or other unsaturated fatty acids) for growth, but these acids are highly inhibitory to growth at higher concentrations (41). Thus in a medium which contains a considerable quantity of free fatty acid no growth occurs. Addition of a synthetic wetting agent and surface-tension depressant, Tween 40, will now permit growth (Fig. 4) simply because it eliminates the toxicity of oleic acid without eliminating its growth-promoting properties (41). Again, if the mechanism of the effect were not known Tween 40 might be considered a true growth factor, although it obviously should not be so considered. These examples are from natural populations. That entirely similar phenomena occur in artificially induced mutants is shown by the isolation of a mutant culture of *Neurospora* which appeared to require sulfonamides for growth. The mutant apparently synthesized *p*-aminobenzoic acid in amounts which indirectly inhibited growth; and by counteracting this action of *p*-aminobenzoic acid the sulfonamides promoted growth of this organism (47). Although a sulfonamide is thus required for growth of the organism, it can scarcely be considered as a true growth factor in the sense that the various compounds of Table 1 are growth factors. Recent reports that certain streptomycin-resistant mutants of bacteria come to require streptomycin for growth may have a similar explanation.

These examples of substances foreign to normal growth processes which come to behave as growth factors can be readily explained once the view is accepted that substances normally present in growing cells, and themselves essential in small amounts for growth, may under certain conditions act as inhibitors of other essential metabolic reactions. It then becomes understandable how such inhibitions can be alleviated either by appropriate normal metabolites (for example, the products of the inhibited reactions within the cell), or by appropriately fashioned anti-

metabolites, which by competing with the toxic metabolite will prevent its toxic action within the cell and thus permit growth.

We have seen that an organism may synthesize a growth factor in one medium which it requires preformed in another. It is also true that
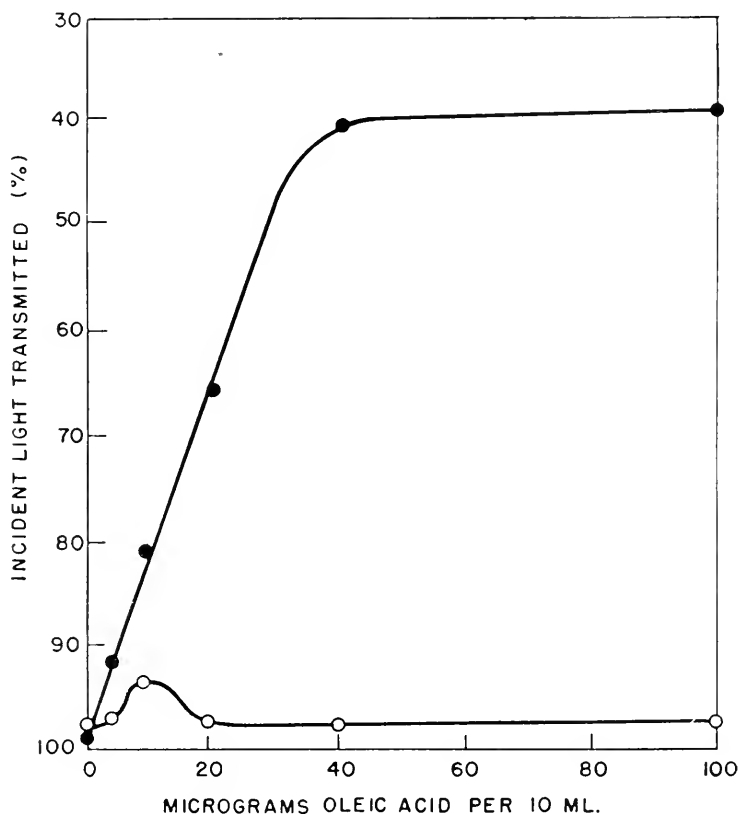
Figure 4. The comparative growth-promoting properties of oleic acid for *Lactobacillus bulgaricus* in the presence (top curve) and absence (lower curve) of Tween 40 (cf. 41).

the magnitude of the requirement for an essential growth factor may vary tremendously, depending upon the composition of the medium, and under conditions such that the variation in the requirement cannot be ascribed to variations in the amount of the growth factor synthesized. Such variations frequently reflect the physiological function of the vitamin, and though more experimental work should be done to establish

the detailed mechanism of such effects, an explanation which undoubtedly holds in many instances may be summarized as follows.

It may be supposed that a bacterium requires a vitamin to carry out a number of catalytic functions necessary for growth. For example, the vitamin may be necessary for synthesis of a number of different essential components (P_1, P_2, P_3, etc.) of protoplasm, from their respective precursors (p_1, p_2, p_3) as indicated in the accompanying diagram.

$$
\begin{aligned}
p_1 &- \quad - \quad \rightarrow P_1 \\
p_2 &- \quad - \quad \rightarrow P_2 \\
p_3 &- \quad - \quad \rightarrow P_3
\end{aligned}
$$
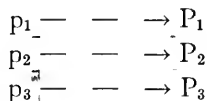
It is logical to assume that if a medium supplies one or more of the products (P_1, P_2, etc.) preformed, the metabolic requirement for the vitamin involved in synthesis of these products might be greatly reduced as compared with the requirement in a medium which did not supply these products.

Such relationships apparently explain the data of Figure 5. It will be
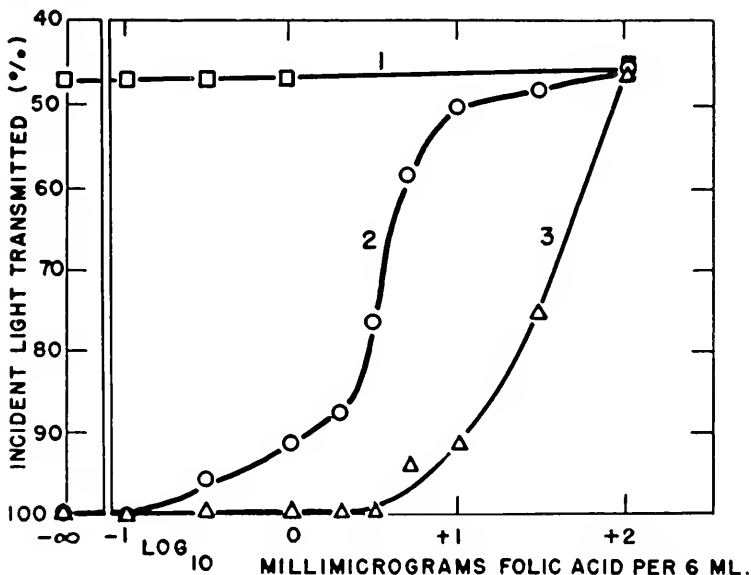

Figure 5. The effects of serine, and of purine bases and thymine on the requirement of *Streptococcus faecalis* for folic acid. Curve 1, serine, thymine, and purine bases present. Curve 2, purine bases and serine present, thymine omitted. Curve 3, purine bases present, thymine and serine omitted (6).

seen that the folic acid requirement of *Streptococcus faecalis* varies remarkably with the nature of the medium. In a medium which lacks serine the folic acid requirement is over ten times that observed in the same medium with added serine. This is interpreted to mean that folic acid is required in relatively large amounts for synthesis of serine, and if serine is supplied preformed, then substantially smaller amounts of folic acid suffice to fulfill its remaining functions (6,11). If to the medium which contains serine, thymine is now added, the folic acid requirement completely disappears (6,38). Under the latter conditions no folic acid can be detected in the cells (38). Apparently the folic acid requirement observed in curve 2 represents that required for synthesis of thymine by these cells, and when thymine is supplied preformed the requirement for this folic acid no longer exists. Traces of the vitamin may still be required for other purposes, but if so, these traces can be synthesized by the cells and are insufficient in magnitude to be detected by present methods of assay.

Several other instances of similar effects are known which will not be given in detail. The vitamin B_6 requirement of lactic acid bacteria, for example, is greatly increased by omitting certain nonessential amino acids from the medium (21) and can be eliminated entirely for some lactic acid bacteria by the addition of D-alanine to a medium which contains a complete assortment of L-amino acids (32,10). Vitamin B_6 is apparently required by these organisms primarily to permit synthesis of amino acids (both L and D) which are essential for synthetic process within the cell, and when all of these amino acids are supplied preformed, the vitamin B_6 requirement is reduced to the point where it can no longer be detected (10). Here, too, analyses have shown that the cells do not synthesize increased amounts (if any) of the vitamin under conditions where they grow without it.

In a wholly analogous fashion the biotin requirement of many lactic acid bacteria can be eliminated by addition of aspartic acid and oleic acid (39,41), and the vitamin B_{12} requirement by appropriate desoxyribosides or reducing agents (12,31). Similar explanations for these results may hold, although further detailed investigations are necessary to establish the mechanism of these effects. It will be apparent from the above examples, however, that a given bacterial species does not necessarily require a fixed and unchangeable assortment of growth factors, but that different combinations, both quantitatively and qualitatively,

may suffice to permit growth by supplying the same deficiencies through different mechanisms.

In the above discussion the attempt has been made to give, at least in outline, the present status of research in bacterial nutrition. The valuable leads that a study of the interrelationships between these nutrients is providing the biochemist should be apparent. The utility of bacteria as test organisms in the microbiological determination of the substances which they require for growth is also well known. One of the most important of the gains to be derived from a study of bacterial nutrition is the recognition of new substances which function as essential growth factors, for such substances have, in the past, always proved to be substances of general importance in metabolism. For example, the recent discovery that D-alanine (10,32) and putrescine or the related compounds, spermine and spermidine (8), are essential for growth of some bacteria lends new importance to these long-known compounds. It had not been previously known whether any of the D-amino acids played essential roles in metabolism; it now appears most certain that in some bacteria, at least, D-alanine is essential for growth (10). Similarly, although putrescine had long been known as a decomposition product of arginine, it was not known to play any essential role in metabolism. Such a role now seems certain from the observation that the compound serves as an essential growth factor for *Hemophilus parainfluenzae* (8).

Elucidation of the chemical nature of the several unidentified growth factors reported as essential for growth of various species of bacteria may similarly be expected to contribute materially to our knowledge of biochemistry and metabolism, for these unidentified substances, like the growth factors of Table 1, are generally distributed in natural materials and undoubtedly have general metabolic significance. Several of the better defined unidentified growth factors are listed in Table 3.

In summary, we have emphasized that the growth factors required by bacteria comprise those compounds, such as the amino acids and the water-soluble vitamins, which are of general importance as essential metabolites in all living organisms. A nutritional requirement for one, several, or many of these growth factors may arise through cumulative, random mutations which result in the loss by the organism of the capacity to synthesize the growth factors which it requires under a given set of environmental conditions. This inability to synthesize a growth factor may result either from loss of one of the enzymes necessary for its

TABLE 3
Some bacteria which require unidentified growth-factors

Organism	Rich Source Material	Reference
Leuconostoc citrovorum	Liver extracts	Sauberlich and Baumann (28)
Lactobacillus bulgaricus	Yeast	Williams, Hoff-Jorgensen, and Snell (42).
*Lactobacillus casei**	Yeast, grass	Guirard, Snell, and Williams (7)
*Streptococcus faecalis**	Yeast, liver	McNutt and Snell (22)
*Tetrahymena geleii**	Liver, yeast (protogen)	Stokstad, *et al.* (40)

*Cross-testing of concentrates of protogen and of the pyruvate oxidation factor of O'Kane and Gunsalus (23) on these various organisms indicate that these various factors are identical (34).

synthesis, or from inhibition of the synthesis at one or another stage by other metabolites normally present within the cell or the medium. The latter occurrence is viewed as being relatively frequent and has been insufficiently emphasized in the past. It permits a ready explanation of the observation that substances such as neopyrithiamine and the sulfonamides, which ordinarily act as growth inhibitors, may with occasional organisms simulate the action of true growth factors. It also explains in many cases the fact that a given organism may synthesize a growth factor in one medium and require it preformed in another. The "sparing action" which certain growth factors may have on the requirement of bacteria for other growth factors has also been discussed. Such sparing actions are evidence for a metabolic relationship between the growth factors involved and are occasionally of such magnitude that the requirement for a specific growth factor may apparently be completely eliminated. Thus in some instances more than a single combination of growth factors may suffice to permit growth of a given organism, although under appropriate conditions each component of each combination may be essential for growth.

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Genetic Aspects of Growth Responses in Fungi

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THE study of growth factors of fungi may be said to have been initiated by the pioneering work of Wildiers with yeast in 1901 (89). Further investigations of the nutritional requirements of this nonfilamentous fungus have contributed outstandingly to the fields of nutrition of both microorganisms and higher organisms. These contributions began with the identification by Eastcott (19) of meso-inositol as one of the bios constituents. The relationship of the nutrition of this microorganism to that of higher organisms first became apparent with the work of Williams (90), who showed that vitamin B_1 was a required growth factor. With this information as a starting point, further investigations with yeast to date have added two additional new vitamins to the list of those important to both microorganisms and higher forms. These are biotin (43) and pantothenic acid (91). Shortly after thiamin had been demonstrated as a growth factor for yeast this vitamin was reported by Schopfer as the first identified essential metabolite required by a filamentous fungus, *Phycomyces blakesleeanus* (70). Investigations with other filamentous fungi initiated by Kögl and Fries (42) and extended since then to a great variety of other fungi (25,67) have fully substantiated the concept that these organisms require essentially the same metabolites as do other forms of life. Strains of fungi are now known which as isolated from nature require the vitamins thiamin, biotin, pyridoxine, inositol, and nicotinic acid. Although not yet identified as a requirement of a filamentous fungus, *p*-aminobenzoic acid is required by certain yeasts. These include *Rhodotorula aurantiaca* (68) as well as strains of *Saccharomyces cerevisiae* (63,64). The available information thus amply supports the generalization that microorganisms, bacteria as well as fungi, and higher organisms have similar requirements for most of the vitamins of the B complex.

Perhaps of equal importance and interest, investigations with various fungi have led to the experimental verification of the genetic basis of the losses in synthetic capacity which in evolution may have led to requirements for exogenous supplies of essential metabolites. Work in this field, initiated with the ascomycete *Neurospora crassa* (6), has been extended during the past several years to a considerable number of other fungi. Mutant strains of a number of fungi deficient in the synthesis of vitamins, amino acids (as listed in Table 1), and nucleic acid constituents, have been produced by treatment with a variety of mutagenic agents including radiation, mustard gas, and other chemicals. With the exception of the imperfect fungus *Penicillium* and the phycomycete *Absidia glauca*, convincing genetic evidence has been obtained that the growth factor

TABLE 1

Requirements of induced mutant strains of fungi

Organism	Vitamin requirements	Amino acid requirements	Mutagens used and reference
Neurospora	all but B_2, choline, biotin*	all but alanine, hydroxyproline	X-ray (7, 45, 81) Ultraviolet (7, 81) S-mustard (39) N-mustard (48, 52, 81)
Ophiostoma	all but B_2, choline, thiamin,* B_6*	for arginine, lysine, methionine	X-ray (25) N-mustard (24) Caffeine (26)
Glomerella	for nicotinic, B_6	for tryptophan, lysine	Ultraviolet (51)
Penicillium	all but pantothenic, B_2	all but alanine, hydroxyproline, serine, glycine, threonine, tyrosine	X-ray, ultraviolet (11) N-mustard (35)
Absidia	for pantothenic, PAB	for histidine, lysine, tryptophan	Ultraviolet (29)
Ustilago	for B_1, nicotinic	for arginine, isoleucine, methionine	Ultraviolet (59)
Saccharomyces	for nicotinic, B_1, PAB,*pantothenic,* biotin*	for lysine, leucine, methionine, phenylalanine, histidine, tryptophan	N-mustard (64) Ultraviolet (62)
Coprinus		for cystine, methionine	N-mustard (23)

*Requirement of parental stock.

deficiencies in all these organisms have resulted from mutation of single genes.

When the various amino acids and vitamins required by strains of fungi isolated from nature and of those isolated following treatment with mutagenic agents are compared, it is seen that requirements found only in mutant strains of fungi include riboflavin, pantothenic acid, p-aminobenzoic acid, and choline. Nicotinic acid has recently been found to be required by *Blastocladia pringsheimii* (16). Folic acid and vitamin B_{12} are the only two B-vitamins which neither strains from nature nor mutant strains have yet been found to require. In regard to the amino acids, very few strains of fungi found in nature fail to grow on inorganic nitrogen or on relatively simple organic nitrogen sources such as asparagine (67,70). *Blastocladia* with a requirement for methionine seems an exception (17). In contrast, mutant strains of fungi have been obtained which have requirements for all of the known amino acids with the exception of alanine and hydroxyproline. Experimentally induced deficiencies for amino acids seem somewhat more frequent than for vitamins. The evidence might be taken to suggest, if mutation in nature is qualitatively and quantitatively compaiable to that induced in the laboratory, that most fungi in nature find themselves in environments where strains with deficiencies for most amino acids and many vitamins would be eliminated from the population. This is in contrast to the occurrence and survival in nature of strains of bacteria which require all of the known vitamins and amino acids with the exception of inositol, choline, alanine, and hydroxyproline. In general bacteria are found in more varied and specialized environments than fungi, and they tend to have lower quantitative requirements for vitamins and amino acids, so that survival of deficient strains would be more likely.

At present comparisons with a given microorganism of the qualitative effects of different mutagenic agents can only be incomplete. Some evidence is available, however, suggesting that with *Neurospora* similar types of mutations are produced by nitrogen mustards, by X-ray, and by ultraviolet light, using a number of different strains as well as a variety of isolation techniques (81). This evidence is consistent with the view that mutation of a given gene is related to the specific lability of that gene rather than to the type of mutagenic treatment. According to this concept a mutagenic agent only accelerates the normal mutation frequency without affecting the quantitative relations between the dif-

ferent gene mutations produced. The types of deficiency mutations found in different fungi under mutational treatment, although similar, have been found to vary somewhat from one organism to another. For example, in yeast the most frequent deficiencies yet reported are for adenine, methionine, leucine, and lysine (62,64). Deficiencies for adenine or hypoxanthine, thiamin, nicotinic acid, and reduced sulfur are most frequent in *Ustilago maydis* (59). Deficiencies for arginine, lysine, and methionine have been most frequently found in *Penicillium* (11). In *Ophiostoma* the most common requirements of mutant strains appear to be for arginine, purines, and pyrimidines (25). In *Neurospora* with the mutagenic agents, strains, and techniques so far used, the most frequent deficiencies have been for methionine, lysine, arginine, and adenine. With *Absidia glauca* the most frequent mutation seems to be for histidine (29). If the specific effects of the various treatments and techniques involved in the isolation of mutants in these fungi has not resulted in selection of particular types of deficiencies, these results would suggest that genes controlling different steps in growth-factor biosynthesis in a given fungus differ in their stability to mutation, and that those genes concerned in particular biosynthetic steps may have entirely different labilities to mutation in different organisms.

Another tentative conclusion regarding the nature of gene control of growth-factor synthesis may well be made at this time. Any technique of production and isolation of mutant strains of a microorganism which does not require carrying the organism through a sexual stage before detection and isolation of a mutant stock, would in theory permit the detection of nutritional deficiencies controlled by extra-nuclear factors, such as those involved in *Paramecium* (77), and in yeast with adaptive sugar utilization (46) and with respiratory systems (20). This condition is met with the fungi listed above, including *Neurospora* in which mutant strains derived from asexual uninucleate microconidia (4) have been isolated (81). Genetic examination of mutants in the fungi so far obtained has failed to indicate the existence of extra-nuclear control of growth-factor or metabolite synthesis. Even in *Saccharomyces cerevisiae*, in which extra-nuclear control of sugar utilization has been suggested, recent work by Lindegren and Lindegren (47) and by Pomper (62) has shown that growth-factor deficiency characters are inherited as if they were characterized by single gene changes. We may, therefore, conclude that in the fungi so far examined, growth-factor deficiencies in mutant

strains, and conversely growth-factor syntheses by normal strains, are controlled by genes located on the chromosomes in the nucleus of the organism rather than by extra-nuclear factors. It might even be suggested that a microorganism which had developed an extra-nuclear system of control of growth-factor synthesis might not survive since such an extra-nuclear system might be more susceptible to environmental modification and accidental loss or destruction.

Although strains of fungi, either of natural origin or mutant strains, are known which require almost all of the B-vitamins which are required by bacteria or higher organisms, rather less is known of the exact function of these vitamins in fungi than in some other organisms. Although there is every reason to believe that the B-vitamins have the same coenzyme functions in fungi as they do in other organisms, direct demonstrations of this in the filamentous fungi are available in only a few instances. That thiamin in the form of cocarboxylase is involved in the respiration of fungi is indicated by the accumulation of pyruvic acid in thiamin-deficient cultures of *Phycomyces blakesleeanus* (32) as well as by the stimulating effect of thiamin on the respiration of homogenized *Sclerotium delphinii* mycelium (61). The situation presumably is similar in *Neurospora* since carboxylase has been demonstrated in this organism (83). Although the involvement of vitamin B_2 in the glucose oxidase system of *Penicillium* has been established (8), and D-amino acid oxidase has been demonstrated in *Neurospora* (37), the participation of riboflavin in this enzyme in *Neurospora* has not yet been established. In *Neurospora* pyridoxal phosphate has been demonstrated to be the coenzyme of the tryptophane synthesizing system (86). Very little evidence is available in regard to *p*-aminobenzoic acid, biotin, and pantothenic acid with the exception of an early report by Giese and Tatum (28) that they were involved in respiratory systems in *Neurospora*. Although biotin has been shown to be involved in sexual reproduction of certain fungi, such as *Sordaria fimicola* (3), only two lines of evidence link the action of biotin in fungi with its functions in other organisms. These are reports that biotin may be spared by oleic acid for *Neurospora* (36), and by aspartic acid for several other fungi (60).

Investigations with mutant strains of *Neurospora* may provide a clue to the nutritional function of inositol. The effect of inositol on this organism was early described by Beadle (5). Limiting amounts of this substance have been found to alter profoundly morphogenetic growth

processes so that a characteristic morphologically limited growth habit is assumed. Although a specific antagonism of inositol action by hexachlorocyclohexane (gammexane) has been suggested by work with yeast (41) and with α-amylase (44), attempts to demonstrate such a relationship in *Neurospora* have been unsuccessful (80). Recent investigations with the pea root (71) and with a variety of bacteria (27) have likewise failed to indicate a specific interrelation between inositol and gammexane. The morphogenetic effect of inositol in *Neurospora* may conceivably be related in some way, at present unsuspected, with the effects of paramorphogenic substances such as tergitol, desoxycholate, and l-sorbose, which have been shown to alter drastically the morphological form of growth of *Neurospora* in a nonhereditary manner (80). The antibiotic produced by *Penicillium griseofulvum* has been shown by Brian to be the "curling factor" which has an effect on the growth of a number of fungi, including *Neurospora* (15), apparently similar to the effects of the paramorphogenic substances mentioned above. Whatever the function of inositol in the growth and metabolism of fungi may be, it is at all events extremely specific for meso-inositol as has been shown recently with *Neurospora* by Schopfer (72).

Considerably more is known in respect to the roles of the amino acids as growth factors in fungi than is true for the vitamins. Most of this information has been obtained from studies with amino-acid-requiring mutants of fungi. These studies in general have substantiated the concepts of comparative biochemistry in that the metabolism and synthesis of amino acids have been found to be similar in fungi, in other microorganisms, and in higher organisms (see 79). For example, we may mention the similarities in the metabolism and synthesis of the sulphur-containing amino acids and of arginine.

In addition these studies with biosynthetically deficient strains of fungi have raised several general points in regard to the significance of amino acids aside from their obvious roles as protein constituents. One of these points concerns the metabolic relationships between certain amino acids and certain vitamins of the B complex. The function of tryptophan as a precursor of nicotinic acid, first suggested by animal experimentation, has been elucidated by studies with mutant strains of *Neurospora*. The findings illustrated in Figure 1 have shown that at least one important route of nicotinic acid synthesis is from tryptophan through kynurenine, perhaps hydroxykynurenine, hydroxyanthranilic acid (12,56), and quino-
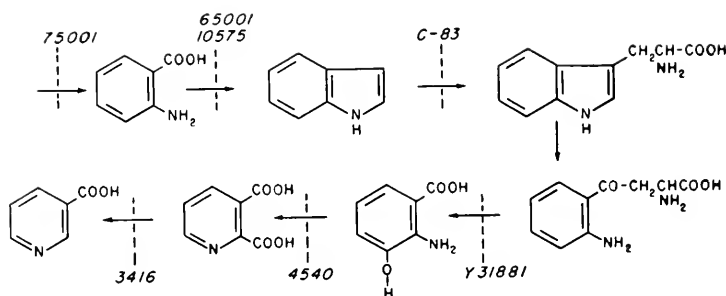
Figure 1. Biosynthesis of tryptophan and nicotinic acid in *Neurospora*.

linic acid (34). Several different mutant strains of *Neurospora* are known which require either tryptophan or nicotinic acid for growth. Strain Y-31881 is unable to use tryptophan but can use hydroxyanthranilic acid which is accumulated by strain 4540. Recently strain 4540 has been shown to utilize quinolinic acid (34) which in turn is accumulated by strain 3416 (9,34). The available evidence indicates that this entire sequence of reactions holds also in the rat (33,34,57).

The other known instance of a relationship between an amino acid and a vitamin is somewhat less direct than that just discussed. This is the relationship established between the aromatic amino acids and *p*-amino-benzoic acid. As illustrated in Figure 2 mutant strains of *Neurospora* are known with single deficiencies for each of the aromatic amino acids and for *p*-aminobenzoic acid. In addition, a single gene mutation has been found to result in the requirement for all four of these substances. This suggests that all four are derived from a common precursor. (See also 58.) Recent investigations have shown that this common precursor
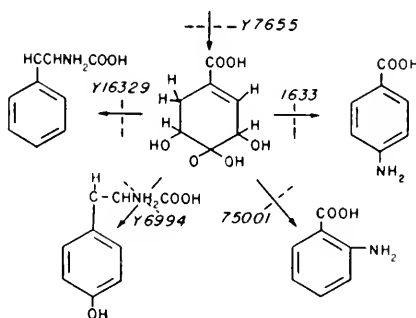

Figure 2. Biosynthesis of aromatic substances in *Neurospora*.

is related to shikimic acid. Shikimic acid will replace all four of these substances for the multiple aromatic mutant of *Neurospora*, Y-7655. That the suggested relationship between these substances is of significance in other organisms is shown by the demonstration by Davis of a corresponding mutant in *E. coli*, which also responds to shikimic acid (18). Fischer has suggested that shikimic acid is derived in the plant directly from hexose sugars (22). In microorganisms the aromatic nuclei of these amino acids and of *p*-aminobenzoic acid may thus be derived from hexoses through a common precursor similar to shikimic acid. Previous results (82) with the *p*-aminobenzoicless mutant strain suggests that in the synthesis of this compound nitrogen is normally introduced into a non-aromatic structure. Since shikimic acid is active only for the multiple mutant, and not for the *p*-aminobenzoicless strain, this structure would seem to be a substance closely related to shikimic acid.

The other important contribution of investigations with micro-organisms is in regard to the roles of many amino acids as biosynthetic precursors of still other amino acids. The interconversion of glycine and serine first indicated in the rat (74) and recently further investigated by Sakami (69), seems also to be true for *Neurospora* and for certain bacteria (31,40). Evidence obtained with mutant strains of *Acetobacter* suggests that glycine is normally formed from serine, which may arise from a nonnitrogenous precursor, since one strain will grow on either glycine or serine and a second strain only on glycine (31). The interconversion of cysteine and methionine by way of homocysteine has been amply demonstrated in *Neurospora* as well as in bacteria (38,76). The role of aminoadipic acid as a precursor of lysine in *Neurospora* (54) represents another extremely interesting example of amino acid interconversions. The role of serine in tryptophan biosynthesis in *Neurospora* is now well known. There seems little question as to the reality of this conversion in view of enzymatic studies which have been carried out (55,86) and in view of the demonstrated incorporation of labeled nitrogen in the form of serine into the tryptophan synthesized by this system (75). The relationships of glutamic acid, proline, and ornithine suggested for the rat (74) have been shown to hold for *Penicillium* (11) and more recently for *Neurospora* (21). Fincham has shown that α-hydroxy-δ-aminovaleric acid is an intermediate in the interconversions of glutamic acid, proline, and ornithine (21). Another extremely interesting series of interrelations is that between the aliphatic amino acids, threonine,

homoserine, and isoleucine. Teas, Horowitz and Fling (85) and Teas (84) have shown that isoleucine and threonine are interconvertible in *Neurospora* and that threonine and homoserine are likewise interchangeable. These relationships and the demonstrated activity of α-aminobutyric acid as a precursor of threonine and homoserine (84) are intriguing relationships which remain to be elucidated. One promising lead in the investigation of these relationships may prove to be the examination of mutant strains of *Neurospora* which require both isoleucine and valine. Continuing the work initiated by Bonner (10), a precursor of isoleucine accumulated by such a mutant strain has been identified as α,β-dihydroxy-β-ethylbutyric acid (1). The results of studies using carboxyl-labeled acetate are consistent with the fairly direct conversion of acetate to the β-ethyl side chain in the isoleucine precursor (2). These results strongly suggest that isoleucine and therefore probably threonine and homoserine are synthesized in *Neurospora* from a common four-carbon precursor.

Of fundamental importance in studies in biochemical genetics in fungi as well as in other organisms is the concept that gene mutation is specifically related to biochemical reaction. Implicit in this concept is the hypothesis that each gene controls a specific biochemical reaction through the action of a specific enzyme (13). The gene and the enzyme are pictured as being specifically related in such a way that gene mutation with a change in the spatial or configurational specificity of the gene results in corresponding changes in enzyme specificity. Mutation of a given gene could then result in failure of the biochemical reaction either if no enzyme at all is produced, or if an enzyme is formed with altered properties such that it can no longer carry out the specific enzymatic function. The examination of particular mutant strains of *Neurospora* for the presence of specific enzymes is of considerable importance in experimentally testing the general concept. In this examination it is, of course, vital to test for differences in the presence or activity of the specific enzyme between the wild-type and the mutant strain.

One of the first examinations of an enzyme system from this point of view was carried out by McElroy and Mitchell (49) in their studies of the adenine-deaminase system of a temperature-sensitive adenine-requiring strain of *Neurospora*. Since this strain could synthesize adenine at temperatures under 28°C. but could not do so at higher temperatures, and since hypoxanthine was inactive for the mutant at higher tempera-

tures at which adenine was required, it was expected that the adenine-deaminase in the mutant might have a different temperature sensitivity from that in the wild-type. McElroy and Mitchell (49) found, however, no demonstrable differences in the enzyme from the wild-type and mutant strains. It was pointed out by these authors that this instance might not represent a critical test of the general concept since adenine synthesis might normally proceed by a reaction other than a reversal of the deaminating system. Another instance which has been examined in *Neurospora* is that of the enzyme involved in pantothenic acid synthesis from pantoic acid and β-alanine. Wagner and Guirard (88) first reported the enzyme which brings about this condensation to be missing in the pantothenicless mutant, and to be present in the wild-type strain. However, Wagner has been able to demonstrate that the enzyme is actually present in the mutant strain (87). A third instance recently investigated is that of the enzyme bringing about the synthesis of tryptophan by condensation of serine with indole, first demonstrated by Umbreit, Wood, and Gunsalus (86). In an investigation of a mutant strain of *Neurospora* which is unable to grow on indole but requires intact tryptophan, Mitchell and Lein (55) reported that this enzyme was not present in the mutant. This example apparently provides excellent evidence in support of the general concept discussed above.

In two examples in *Neurospora*, which may prove to be pertinent, carboxylase has been demonstrated in a strain which requires acetate or ethanol for growth, and asparaginase has been found in a strain which has a specific requirement for asparagine (83). Although generalizations from relatively few specific examples are always dangerous, the instances just discussed might indicate that gene mutation may affect metabolism in some microorganisms by modifying intracellular conditions so that the enzyme is normally inoperative; or alternatively by leading to the production of a specific enzyme inhibitor, and thus indirectly to the production of an enzyme inactive *in vivo*. Gene mutation, therefore, in at least some instances, may modify a biochemical reaction in the cell not by direct modification or elimination of a particular enzyme, but rather by controlling its *in vivo* activity in some manner not as yet understood.

Some additional suggestions as to the nature of the gene-enzyme relation may come from examination of the three general types of behavior of biochemically deficient strains of fungi which have been

described. In mutant strains of *Neurospora* one type of behavior typical of what has been termed an "absolute deficiency" is characterized by the failure of the mutant strain to grow in the absence of the required supplement, or by its being capable of a very slight amount of growth which does not continue after the stored material in the inoculum has been exhausted. Most of the mutant strains used in biochemical studies appear to belong in this group. A second type of behavior is that shown by strains with so-called "partial deficiencies" which are capable of growing on minimal medium at a more or less constant rate which is less than that of the wild-type or of the mutant on supplemented medium. The behavior of these mutants has been attributed to a modification of the enzyme systems such that the limiting reaction can proceed to a certain extent but at a rate insufficient to permit optimal growth (53). A third type of behavior is typified by a strain which on inoculation into minimal medium grows very slowly for a considerable period of time. Finally, after a lag period of variable duration, its growth begins to improve and may approach or even reach the wild-type rate. In some cases this growth in minimal has been shown to be due to gene reversion, as in the inositoless mutants investigated by Giles and Zimmer (30). In other cases the phenomenon seems not to be due to gene reversion and has been termed "adaptation" (14). In these instances the ability to grow in the absence of the specific supplement is lost on passing through the asexual spores, that is the conidia, or through the sexual spores, the ascospores. Reasonable interpretations of this phenomenon of adaptation are those of the development of an alternative route of synthesis by-passing the genetically blocked reaction, or of the reconstruction of the genetically blocked reaction through the production of an alternative adaptative enzyme system. Similar behavior patterns have been described in other fungi, for example in pleomorphic cultures of the imperfect fungus *Trichophyton* (66), in which the genetic basis of the phenomenon cannot be directly examined.

The phenomenon of adaptation in *Neurospora* has been further examined recently in a few strains in which gene reversion has been rigorously excluded by genetic methods. Regnery (65) has examined the adaptive behavior of leucineless 47313, and Tatum and Garnjobst (83), the adaptive behavior of tyrosineless Y-6994. With these mutants it has been found that mycelium which has reached a wild-type rate of growth, either after a prolonged lag period on minimal medium or even

in response to optimal concentrations of the required substance, is completely independent of an exogenous supply of the growth factor since it can grow indefinitely at maximal rate on minimal medium from mycelial transfers. The adaptive behavior of these mutants is not carried through the conidia, and the cultures must again pass through a lag phase on minimal medium. Although a differential storage in the conidia of the required factor may differentiate the mutant and wild-type, this does not seem likely. In contrast to wild-type conidia or ascospores which germinate and grow immediately on minimal medium, ascospores of the leucineless mutant send out germ tubes but fail to grow further on minimal medium. Ascospores of the tyrosineless strain initially grow slowly even on supplemented medium. Although much additional information is needed before a final conclusion can be reached, existing information suggests that as the result of mutation the enzyme systems involved in biosynthesis of these factors are lost in these mutants during the return of the cytoplasm to a resting state in conidiation or ascospore production, and that the reconstitution of these systems depends upon and always accompanies growth. We may therefore have in these adapting strains examples of gene control of a biochemical reaction by altering the efficiency of initiation of the synthesis of an enzyme rather than by affecting enzyme production either quantitatively or qualitatively as suggested in the cases of the complete or partial blocks as discussed above. Strains which behave in this manner may represent the closest approximation to an extra-nuclear control of enzyme production yet found in *Neurospora*, since the maintenance of the enzyme activity in the cytoplasm would seem to be to a certain extent independent of the gene.

Studies of the nutrition of fungi have gone through two general phases. First, the identification of specific growth requirements leading to culture of the organisms on media of known chemical constitution, with the substantiation of the tenets of comparative biochemistry that fungi require the same factors, amino acids and vitamins, as do other organisms. Second, the experimental demonstration of the gene control of biosynthesis of these factors by means of experimental gene mutation with consequent biosynthetic deficiencies. Detailed biochemical study of strains with such induced deficiencies has added significantly to biochemical knowledge of vitamin and amino acid syntheses and interrelations. It may be predicted that future investigations will continue

to contribute valuable information in these fields. Even more important, however, may prove future contributions of research with fungi along a number of lines discussed in this paper. It seems probable that these may lead to a better understanding of the nuclear and cytoplasmic factors involved in enzyme production, specificity, and activity, a problem of fundamental importance in all fields of biology.

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Vitamin and Amino Acid Requirements for the Growth of Higher Plants

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Vitamins

Oₙₑ of the difficulties in discussing the vitamin requirements of higher plants is to define the term vitamin, and to delimit the substances to be considered.

In the authorized English translation of the second German edition of his book on vitamins, Funk (15) approved Willaman's (62) definition which is as follows: "Vitamins constitute a class of substances, the individuals of which are necessary for the normal metabolism of certain living organisms but which do not contribute to the mineral, nitrogen or energy factors of the nutrition of these organisms." This definition might be interpreted to include any organic substance which has the following characteristics: it is an essential metabolite, it functions in some other way than as a source of energy or as a major constituent of the plant or animal body, and some individual organism must be supplied with it from without for normal function and development.

Although by usage the word vitamin has special connotations which limit its application to a more or less well-defined group of substances, the distinction between vitamins, hormones, auxins, growth substances, and cofactors, has tended to disappear. With some exceptions the distinction between the vitamins required by animals and those required by plants has also become less marked. In fact, it is preferable to consider growth requirements from the standpoint of essential metabolites rather than to limit consideration to vitamins, amino acids, purine and pyrimidine bases, or any other particular group of substances. Students of the growth requirements of any group of plants find it advantageous

to be acquainted with those of other groups, both plants and animals, and to include in their investigations not only vitamins as such but amino acids, hormones, and other substances which have been demonstrated to play a significant role in plant and animal metabolism.

Intact Plants.—Whether we consider vitamins in the narrower sense, that is, the B vitamins, vitamin A, C, D, E, and so on, or in the broader sense, intact higher plants have no vitamin requirements. This is demonstrated by their ability to grow in a mineral medium with no organic supplements. The evidence at hand indicates also that with some possible exceptions and under special circumstances, higher plants have no partial deficiencies for vitamins, that is, their growth is not improved by the addition of vitamins to the medium. They appear to synthesize adequate quantities of all the vitamins they need. Bonner and Bonner (10) in a recent review of this subject say that thiamin is not a limiting factor in the growth of most species of higher plants and can become a limiting factor only for a few species or under particular environmental circumstances. The same conclusion appears to hold for the effects of niacin, pyridoxine, adenine, pantothenic acid, riboflavin, and other similar substances for which growth increases have been reported by one investigator or another.

Excised Parts.—Although the intact higher plant is self-sufficient as far as vitamins are concerned, isolated parts are not. They may evidence heterotrophism for various vitamins.

Excised roots of a number of species have been demonstrated to have complete or partial, deficiencies for thiamin, pyridoxine, and niacin. Stem tissues and the tissues of storage organs require indoleacetic acid (IAA) or its equivalent, and in some instances (willow and hawthorne) pantothenic acid and biotin. Thiamin and cysteine are not essential but may improve growth (18). The contrast between the relation of intact plants to vitamins and that of isolated organs or tissues emphasizes the interdependence of the parts of a higher plant and has led to the thesis that the B-vitamins may be considered to be plant hormones (10).

Excised Roots.—We know more perhaps about the vitamin relations of excised tomato roots than about those of the roots of any other plant. White (58) first succeeded in obtaining unlimited growth of tomato roots in a solution of mineral salts, sugar, and dried yeast. Robbins and Bartley (43) demonstrated that excised tomato roots would not grow unless the yeast extract was included in the sugar-mineral salt solution. They found

thiamin to be the essential factor in the dried yeast (47). This confirmed the earlier suggestion by Robbins (40,41) that the failure of an excised root to continue growth when repeated transfers of the root tip are made is because the seedling root contains some materials derived from the seed other than water, mineral salts, sugar, and free oxygen, which are necessary for continued growth and cannot be synthesized in solution cultures from the materials supplied.

Later Robbins and Schmidt (48) demonstrated that tomato roots have a partial deficiency for pyridoxine, and Bonner (5) found some clones to have complete deficiencies for thiamin and for pyridoxine and partial deficiencies for niacin. All three vitamins show a high degree of specificity.

The roots of a substantial number of species have been cultivated in excised condition, and for many of them potentially unlimited growth has been obtained. Among these are *Acacia melanoxylon*; alfalfa (*Medicago sativa*); aster (*Callistephus chinensis*); buckwheat (*Fagopyrum esculentum*); carrot (*Daucus carota*); celery (*Apium graveolens*); chicory (*Cichorium* sp.); red clover (*Trifolium pratense*); white clover (*Trifolium repens*); sweet clover (*Melilotus alba*); cotton (*Gossypium hirsutum*); *Crepis rubra*; Jimson-weed (*Datura stramonium*); mustard (*Brassica nigra*); pea (*Pisum sativum*); *Petunia violacea*; radish (*Raphanus sativus*); soybean (*Glycine soja*); tobacco (*Nicotiana Tabacum*, *N. langsdorfii*); tomato (*Lycopersicon* sp.); and vetch (*Vicia* sp.) (6,10,60).

A number of the above have been cultivated only in a sugar-mineral salt medium supplemented with yeast extract and their exact vitamin requirements have not been defined. For the others, thiamin, pyridoxine, and niacin are the only vitamins which have been demonstrated to be of importance. The excised roots of one species, flax, make limited growth through an indefinite number of transfers in the absence of any added growth substance. The addition of thiamin, however, increases the growth substantially. White clover is able to grow indefinitely in the absence of thiamin but requires niacin. Of 12 additional species, all require thiamin. Four species require thiamin and pyridoxine, but their growth is increased by the further addition of niacin. The results to date emphasize the importance of thiamin as a vitamin requirement for the growth of excised roots, but, depending upon the clone or species used, all possible combinations of complete and partial deficiencies for the three vitamins occur.

There is, however, a substantial number of species for which the conditions for unlimited growth of excised roots are unknown. Among these are included: *Bauhinia purpurea*; beet (*Beta vulgaris*); broccoli (*Brassica oleracea*); *Bryophyllum calycinum*; cabbage (*Brassica oleracea*); corn (*Zea Mays*); cucumber (*Cucumis sativus*); eggplant (*Solanum Melongena*); grape (*Vitis* sp.); grapefruit (*Citrus maxima*); kohlrabi (*Brassica oleracea*); lemon (*Citrus Limonia*); lettuce (*Lactuca scariola*); lupine (*Lupinus* sp.); orange (*Citrus sinensis*); *Parthenium argentatum*, *Poa* sp.; *Poinciana gilesii*; potato (*Solanum tuberosum*); rice (*Oryza sativa*); *Simmondsia californica*; *Sterculia diversifolia*; *Thuja orientalis*; 3 species of tobacco (*Nicotiana glutinosa*, *N. rustica*, *N. sylvestris*); and *Wisteria sinensis* (6,10,60). Potentially unlimited growth has not been obtained for the roots of any monocot or cucurbit, and the roots of many woody plants have proved to be refractory.

Other conditions than vitamin requirements may be important in determining unlimited growth of excised roots. This is suggested by the peculiar results obtained by Robbins and Maneval (45) with lupine, and by Bonner (6) with the roots of *Sterculia diversifolia*, *Bauhinia purpurea*, and *Wisteria sinensis*. In these cases out of many roots the majority failed to grow under excised conditions, but a single individual might show very substantial growth. McClary (31) has reported that he could obtain unlimited growth for the excised roots of a hybrid corn on an agar medium containing sugar and mineral salts and has suggested that physical factors are determinative rather than vitamin supplements or other growth substances. Bonner was unable to confirm McClary's observations on the unlimited growth of corn roots, and we have not been successful in obtaining unlimited growth.

Excised Stem Tips.—Efforts to cultivate excised stem tips are complicated because, as a rule, the stem tips develop roots and the investigator is then dealing with an entire plant. Dodder and asparagus stem tips are relatively free from this complication. Loo (29) found that seedlings of dodder grew well during the first week but ceased to grow in the third week in a sucrose-mineral salt solution in diffuse light. Stem tips of dodder were kept alive in diffuse light in a sucrose-yeast extract medium for 10 months through a series of transfers. The stem tips developed considerable chlorophyll. In the dark, however, they failed to maintain their growth even in the sucrose-yeast extract medium. Loo (27,28) also cultivated isolated stem tips of asparagus in diffused light on nutrient

media containing mineral salts and sugar. On this medium the stems grew actively through repeated transfers extending for 22 months. In the dark, however, growth approached zero after a few transfers. The growth requirements for excised stem tips cultivated in the dark are still obscure. The slow absorption of sugar and other materials by the excised stem may be an important factor.

Excised Tissues of Stems or Storage Organs.—Nobécourt, Gautheret, and others have obtained unlimited growth of portions of stems, storage organs, or callus of a considerable number of species of plants. These include among others: carrot (*Daucus carota*); chicory (*Cichorium Intybus*); *Cissus discolor*; grape (*Vitis incisa, V. vinifera* var. *Aramon., V. Coignetiae, V. Davidii*); hawthorne (*Crataegus monogyna*); Jerusalem artichoke (*Helianthus tuberosus*); *Parthenocissus tricuspidata*; *P. Hederaefolia; Rubus fructicosus*; *Scorzonera Hispanica*; snapdragon (*Antirrhinum majus*); and turnip (*Brassica campestris*) (18).

Indoleacetic acid or its equivalent is of primary importance for the growth of these tissues. It represents an essential factor for many of them. Some (carrot, grape, Jerusalem artichoke, salsify (*Tragopogon*), *Scorzonera*, and turnip) grow slowly without the addition of IAA to the medium (17,18), but their growth is much more rapid when the medium is supplemented with this substance. Apparently they synthesize a small amount of IAA but insufficient for maximum growth. Cysteine and thiamin improve the growth of some of these tissues; the callus of *Salix caprea* and the stem tissues of hawthorne (*Crataegus monogyna*) are reported to require pantothenic acid and biotin in addition to IAA (18,19).

The relation of IAA to the growth of excised tissues of stems and storage organs suggests that this substance or its equivalent acts as an essential metabolite which is not synthesized in adequate quantities by the isolated tissues of stems and storage organs. Its relation to these tissues appears to be of much the same order as the relation of thiamin, pyridoxine, or niacin to the growth of excised roots.

Plant Embryos.—Considerable attention has been devoted to the culture of plant embryos by Blakeslee (2), Hannig (20), LaRue (26), Tukey (55), van Overbeek (56), and others. Hannig more than 40 years ago cultivated embryos of some of the *Cruciferae*. Immature embryos evidenced little development after removal from the seed; older ones were grown to maturity. In general, attention has been devoted

to the conditions necessary for the development of immature embryos and of those which fail to mature in the seed. The most successful efforts in defining the growth requirements for immature embryos are those of Sanders and Burkholder (51) discussed later in this paper. Unfortunately their investigations do not establish the vitamin requirements of the embryos, if any. From scattered observations we know a little of the vitamin requirements of the embryos of higher plants. Kögl and Haagen-Smit (23) found that biotin and thiamin increased the growth of pea embryos freed of their cotyledons; Bonner *et al.* reported that panto-thenic acid (9), ascorbic acid (3), or niacin (4) benefited pea embryos; Noggle and Wynd (37) state that niacin induced good germination and excellent development of *Cattleya*.

Tumor Tissue.—Plant tumors and "accustomized" tissues in contrast to normal tissues excised from stems or storage organs grow in a sugar-mineral salt medium with no supplements. They do not require an external supply of IAA; their growth may be improved by thiamin. Judging from the evidence presented by Gautheret and his colleagues (24,34), "accustomized" tissue and at least some kinds of plant tumor tissues have an enhanced power to synthesize IAA.*

An obvious explanation for the difference in growth requirements of normal tissue as compared to "accustomized" tissue or tumor tissue is that in becoming tumerous or in becoming "accustomized"† the original tissue has mutated (14) and developed a strain with greater ability to synthesize IAA. It is impossible to say whether the mutation is nuclear or cytoplasmic.

Somatic mutations (or saltations) resulting in increased power of synthesis are known elsewhere in the plant kingdom and there is no a priori reason why such mutations, spontaneous or induced, should not occur in higher plants. We have studied a strain of *Fusarium avenaceum* which evidenced a complete deficiency for biotin. A spontaneous mutant isolated from this strain was able to synthesize its own biotin (44). Many species of fungi produce spontaneous somatic mutants with enhanced powers of growth. The greater vigor of mutants (pleomorphisms) of some of the dermatophytes as compared to the strains from which they

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*Riker, Henry, and Duggar (38) found no more auxin in crown-gall tissue than in normal tissue.

†See the observations of Morel (33).

are derived appears to be associated with an increase in the efficiency of nitrogen metabolism (46).

Some Unsolved Problems.—Our knowledge of the relation of vitamins to higher plants is still far from complete. I have called attention to the substantial number of species for which the conditions necessary for unlimited growth of excised roots have not yet been defined. The same comment applies to excised stem tissues.

The evidence for the effects of vitamins on intact higher plants is confused and in most instances unconvincing. However, the growth requirements of such parasites as dodder or saprophytes like Indian pipe have not been defined. The effects of the additions of natural organic supplements on the growth of the *Lemnaceae* (50), the relations of mycorhiza to some higher plants, the increased growth frequently associated with polyploidy, the phenomenon of hybrid vigor, genetical dwarfs, flowering and photoperiodism, all deserve further investigation from the standpoint of partial deficiencies of essential metabolites including vitamins.

We know very little of the relations of higher plants to pteroylglutamic acid or to vitamin B_{12}. One green plant, *Euglena*, is heterotrophic for vitamin B_{12} (22). The presence of this vitamin in higher plants has not been demonstrated, and no instance of its importance for higher plants has been reported.

The difference in vitamin requirements of tissues derived from stems or storage organs and those of excised roots deserves further investigation. For the former, an external supply of IAA is the critical factor; for the latter, thiamin, pyridoxine, or niacin include the essential supplements. This distinction applies even when the excised roots and the excised stem tissue are derived from the same species. It exists also when the requirements of tissue obtained from a storage root are compared with those of a seedling or fibrous root of the same species. Bonner (5) obtained unlimited growth of seedling carrot roots in a sugar-mineral salt solution supplemented with thiamin and pyridoxine; both vitamins were essential. Tissue from the storage root of carrot requires for continuous and vigorous growth only IAA or its equivalent; other supplements are not necessary. Nobécourt (36) observed that fibrous roots which develop from the tissue isolated from the storage root do not grow in the sugar-mineral salt medium supplemented with IAA when

detached from the tissue. They require thiamin.* This suggests that the growth requirements for cambial meristem are different from those of the apical root meristem.

When we include a consideration of the vitamin requirements of the meristems of such tissues as plant tumors, it seems likely that the metabolism of various meristems in or from the same plant is not identical. This might be assumed from the differences in the cellular elements and tissues formed by the various types of meristem. However, the determination of the growth requirements of these meristems defines some of the specific differences in their metabolism. How these differences come about, what elements in the cells are responsible for them, their implications for differentiation or morphogenesis and for abnormal growth, are questions for further research.

Methods of Investigation.—Methods of investigation in this field are exacting and the interpretation of results requires an appreciation of certain general concepts. Some considerations which should be borne in mind are as follows:

(1) Species differ in their requirements. Clones of the same species and even meristems from the same plant may exhibit different responses.

(2) Vitamin deficiencies may be complete or partial, single or multiple, absolute or conditioned, permanent or temporary.

(3) Because of the relation of microorganisms to vitamins, experiments under nonsterile conditions must be reviewed with caution. When vitamins are added to soil or sand cultures, effects noted on higher plants may be the result of their action on the microflora.

(4) Reserves of vitamin in the seed or excised portion of a plant may compensate for a deficiency in the medium. Successive passages in a vitamin-free medium are advisable. White (60) has suggested ten passages as a criterion for determining potentially unlimited growth.

(5) In experiments involving vitamins, the basal medium should be adequate in all other respects and approach as nearly as possible that most suitable for the organism in question.

(6) Environmental conditions, including hydrion concentration, temperature, and salt concentration, may affect vitamin requirements.

(7) Because of the minute amounts of vitamins which are effective and because of their wide distribution in products of natural origin,

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*The difference in the requirements of excised fibrous carrot roots as observed by Bonner and Nobécourt may be due to the use of different varieties of carrot.

special attention must be given to cleanliness of glassware and other utensils and to purity of chemicals. Effective quantities of one or more vitamins may be present in the carbohydrates (maltose of adequate purity is especially difficult to obtain), in agar, in gelatin, in cotton, cheese cloth, asparagine, or any other product of natural origin. We have found some samples of the vitamin thiazole contaminated with the vitamin pyrimidine.

Amino Acids

As far as is known higher plants have no amino acid requirements in the sense that they are heterotrophic for a particular amino acid or group of amino acids and will not grow unless supplied from without with one or more of these metabolites. Higher plants are able to synthesize all the amino acids included in their cell substance from inorganic nitrogen, for example, nitrates or compounds of ammonia. In fact, it is probable that any cell of at least some species of higher plants can construct amino acids from inorganic compounds of nitrogen. This is suggested by the demonstration that some kinds of excised roots (42,7) and stem tissues (12,27,39) grow in media in which nitrates are the only source of nitrogen.

With the possible exception of embryos (51) and perhaps some stem tissues (16), higher plants appear to synthesize amino acids from inorganic nitrogen as rapidly as they can be used in the plant's metabolism. The burden of evidence indicates that better growth is obtained with inorganic nitrogen than with any amino acid or mixture of amino acids. Furthermore, most investigations fail to demonstrate beneficial effects from the addition of one or more amino acids to a solution containing inorganic nitrogen if the plant is adequately supplied with carbohydrate through photosynthesis or otherwise.

I say, as far as is known, higher plants have no amino acid requirements. Not all of them, of course, have been investigated, and it is possible that such parasites as dodder or saprophytes like Indian pipe, have amino acid deficiencies. This question cannot be answered until these plants are successfully grown on a medium of known composition. Neither can we assert that all roots grow as tomato roots do with nitrates as the only source of nitrogen. It is conceivable that some of the roots which have not been successfully cultivated in excised condition may have amino acid requirements. The same may be said for stem tissues.

We can summarize briefly by saying that, with the exception of embryos, investigations generally indicate that higher plants have no complete or partial requirements for amino acids.

This does not mean, however, that higher plants are unable to absorb amino acids as such and utilize them. Numerous investigations on the direct utilization of organic nitrogen by higher plants beginning with those of Lutz (30) in 1898 have demonstrated that some amino acids supplied under sterile conditions can be absorbed and utilized under some circumstances. There is disagreement, however, as to which amino acids can be assimilated by intact plants and the conditions under which they are utilized are ill-defined. Hutchinson and Miller (21) in summarizing the literature in 1911 said that more or less satisfactory evidence of assimilation had been obtained for leucine, aspartic acid, asparagine, and tyrosine. The gains of nitrogen were, however, generally very small, and in many cases negative results were obtained. With some modification in the list of specific amino acids, the situation nearly 40 years later is about the same.

For almost any amino acid for which utilization is claimed, other evidence showing that it is not utilized or is toxic can be cited. Aspartic acid, for example, is reported by Molliard (32) to be assimilated by radish; Beaumont *et al.* (1) obtained negative results with tobacco; Hutchinson and Miller (21) found it to be a fair source of nitrogen for peas; Tanaka (54) states that it is not utilized by *Sisyrinchium*; Virtanen and Linkola (57) found it to be assimilated by peas and clover but not by wheat and barley for which it was injurious. Brigham (11) states that asparagine is superior to nitrates for dent corn; Beaumont *et al.* (1) found asparagine to be a fair source of nitrogen for tobacco; Steinberg (53) reports it to be quite toxic for tobacco. White (59) in 1937 concluded that 9 amino acids were essential for the growth of excised tomato roots and called attention to the close correspondence between the amino acids essential for the growth of rats, for diphtheria bacilli, and for tomato roots. Two years later (61) he reported that the 9 essential amino acids could be replaced by glycine which was not included in the original group. Bonner (7) and Day (13) were unable to demonstrate beneficial effects of glycine. We have grown excised tomato roots through 137 passages extending over almost 13 years in a medium in which the only organic constituents were sugar and thiamin and the only source of nitrogen was nitrate.

The confused and unsatisfactory status of our information on the relation of amino acids to higher plants may be ascribed in part to a variety of causes among which are the following.

Sterility. It is probably not necessary to emphasize the importance of maintaining sterile conditions. Any research with amino acids under nonsterile conditions must be viewed with suspicion if the purpose is to determine the effects of the amino acid rather than of its decomposition products. Unfortunately, a good deal of the earlier work on the influence of amino acids was carried out under nonsterile conditions.

Purity of Amino Acids. The purity of the sample of acid used is always a matter of concern. Proline, for example, materially influences the action of hydroxyproline on dermatophytes, and many of the so-called pure samples of the latter compound contain sufficient proline to affect the results materially (46). In addition to the purity of the individual amino acid, attention must be given to its optical form. The natural form may act differently from the unnatural or from mixtures of the two.

Secondary Effects of Amino Acids. In evaluating the action of amino acids per se, consideration must be given also to their buffer action. We found, for example, the favorable effect of glutamic acid on the gametic reproduction of *Phycomyces* to be due to its buffer effects (49). Amino acids may combine with heavy metals (52) reducing the toxicity of a medium or lowering the amount of a minor essential element below the optimum. Nielsen and Johansen (35) found that asparagine materially reduced the toxicity of copper for *Rhizobium radicicola* probably by forming a copper complex. Amino acids may react during heat sterilization with other constituents of the medium, especially dextrose (63). Lankford (25) reports that the sterilization of glucose with amino acids produces effects on lactic acid bacteria quite different from those obtained when the amino acids and glucose were separately sterilized.

Specificity of Action. Another complicating factor is that the effect of an amino acid may vary with the plant species. Hydroxyproline is quite toxic for a number of dermatophytes but is relatively harmless, even beneficial, for other fungi (46). Virtanen and Linkola (57) report that peas and clover use both optical forms of aspartic and glutamic acids well for their nitrogen nutrition, but that aspartic and glutamic acids do not function as nitrogen sources for wheat and barley. In fact, aspartic acid appears to interfere with the growth of wheat in media containing nitrate or ammonium salts. Legumes and grasses seem, therefore, to exhibit entirely different behavior toward aspartic or glutamic acid.

Single Amino Acids versus Mixtures of Amino Acids. The results obtained with microorganisms and the excellent investigation by Sanders and Burkholder (51) on *Datura* embryos emphasize that mixtures of amino acids may have substantially greater beneficial effects than any single amino acid. Further work on amino acid requirements of higher plants should include a consideration of the balance between amino acids.

Embryos versus Adult Plants—Parts versus Whole Plants. Attention must be given to the stage of development of a plant and to the results obtained with root or stem tissue as compared to the intact plant.

Carbon Content of Amino Acid. The possibility that an amino acid may be

effective because it furnishes a carbon configuration rather than because of its nitrogen content is another factor to be considered.

Miscellaneous Factors. It is hardly necessary to mention that temperature, light intensity, the pH of the medium, and other important environmental factors may affect the action of amino acids. Riker and Gutsche (39) have emphasized the significance of the concentration of an amino acid in determining its effects.

Importance of Research on Amino Acids.—Interest in the relation of higher plants to amino acids has extended in various directions. There has been a substantial amount of research on the possibility that organic fertilizers owe their effects in part to the absorption of nitrogen in organic form including amino acids. The burden of evidence indicates that except in particular circumstances the direct absorption of amino acids from organic fertilizers is of little or no importance. The inhibitory action of particular amino acids and the balance between them has also received attention. It is suggested that an excess of free amino acids in protein metabolism may be responsible for the symptoms of specific plant diseases, that is, isoleucine and frenching in tobacco (53); that amino acids may act as growth-regulating substances (39) or as cofactors for auxin (8). The amino acid balance is considered of importance in determining the growth pattern of a plant (57) and in the problem of differentiation, dormancy, and interspecific crosses (51).

It seems odd that there is so little evidence that amino acids are as good or better for higher plants than inorganic nitrogen. If amino acids are synthesized from inorganic nitrogen and then condensed stepwise through proteoses, peptones, and polypeptides to protein, one would expect that suitable mixtures of amino acids should be as effective or more effective than inorganic nitrogen. Is the evidence incomplete? Are amino acids absorbed and translocated too slowly, or do proteins arise by the condensation of units of different and perhaps simpler form than amino acids?

Although substantial progress has been made in our knowledge of the vitamin and amino acid requirements of higher plants, it is evident that what we now know is a beginning only. Further work on intact plants, embryos, and excised tissues aimed at defining growth-substance requirements and including all types of essential metabolites will doubtless elucidate many of the questions which are still unanswered.

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